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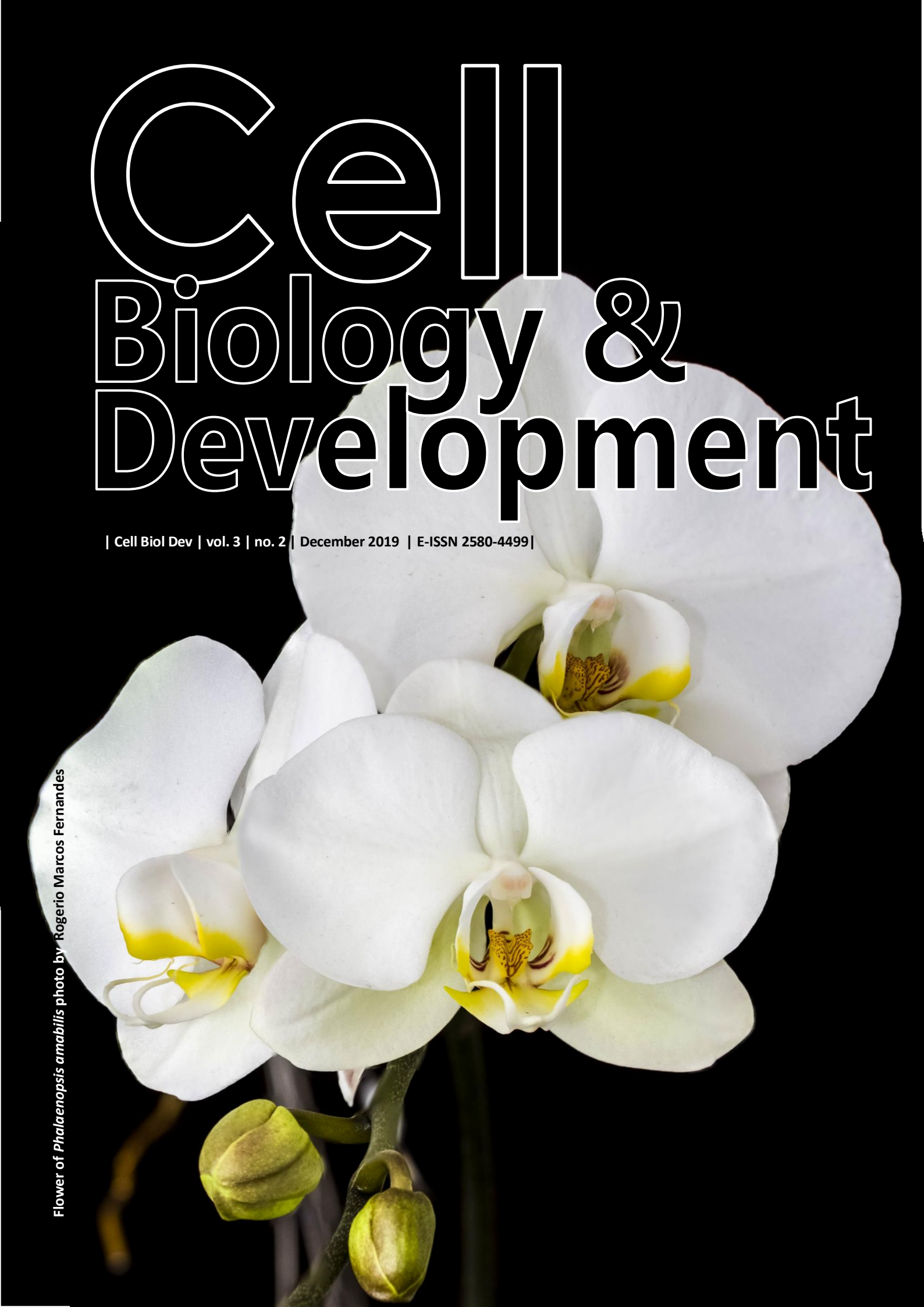

Flower of *Phalaenopsis amabilis* photo by Rogerio Marcos Fernandes

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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

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Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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The effect of auxin and auxin inhibitor application on induction and proliferation of protocorms in immature fruit *Phalaenopsis amabilis* in vitro culture

AGUSTINA PUTRI CAHYANINGSIH[1], ARI PITOYO[2,♥], SOLICHATUN[2]
[1]Bioscience Graduate Program, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel./fax. +62-271-663375, ♥email: aripitoyo@staff.uns.ac.id

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Abstract. *Cahyaningsih AP, Pitoyo A, Solichatun. 2019. The effect of auxin and auxin inhibitor application on induction and proliferation of protocorms in immature fruit* Phalaenopsis amabilis in vitro *culture. Cell Biol Dev 3: 49-55.* Auxin is a hormone that plays an important role in the ovulum development of moth orchid *Phalaenopsis amabilis* after pollination. Using immature fruit 60 days after pollination as explants are presumed to cause cessation of endogenous auxin distribution due to early fruit harvesting. The research was aimed to investigate the effect of exogenous auxin addition on induction and proliferation protocorm in moth orchid immature fruit explant through in vitro culture. This study used NAA (α-Naphthaleneacetic acid) as auxin in MS medium with 4 treatments: (i) control, (ii) NAA 1 ppm, (iii) NAA 5 ppm, and (iv) TIBA (2,3,5- triiodobenzoic acid) 5 ppm. Observed data include explants' color change, development, anatomy after planting, and protocorm development. Morphological and anatomical data were presented descriptively, and protocorm measurement data were analyzed using One Way ANOVA and followed by a DMRT test of 5% level. The results showed that the culture of immature fruit *P. amabilis* 60 DAP with the addition of NAA 1 and 5 ppm in immature fruit culture induced protocorm. Still, the protocorm was not able to develop further. Without adding exogenous hormone-induced protocorm formation, immature fruit culture developed some protocorm to form leaf primordia. Next, adding TIBA as auxin transport inhibitor, 5 ppm induced protocorm and developed all protocorms to form leaf primordia.

Keywords: Auxin, immature fruit culture, *Phalaenopsis amabilis*, protocorm

Abbreviations: DAP: Days After Planting; NAA: α-Naphthaleneacetic acid; TIBA: 2,3,5- triiodobenzoic acid; FAA: Formalin-Aceto-Alcohol

INTRODUCTION

Phalaenopsis amabilis (L.) Blume or *moon orchid* (Indon.: *anggrek bulan*) is one of the flowers that are popular among lovers of ornamental plants and are still primadonna, and the economic value is quite high. Consumer interest in moon orchids also leads to high production to meet market demand. However, the high interest in the moon orchid does not seem to balance its productivity. That is possible because of obstacles to the propagation of orchids. The unique structure in orchid flowers makes it difficult to pollinate; naturally, seeds produced from pollination are seeds that do not have an endosperm, so germination must be assisted by mycorrhizal (Bazand et al. 2014). Therefore, various problems encourage the research development to optimize orchid cultivation methods.

The plant tissue culture technique is an alternative method of propagation that can overcome the constraints of orchid propagation with a high rate of propagation success (Balilashaki et al., 2014). Propagation in orchids is not only demanded in terms of quantity but also terms of quality. Vegetative propagation carried out has not been able to be used to get orchid plants with superior variations. In-vitro generative propagation can be used to solve the limitations of vegetative propagation.

In vitro generative propagation is one solution to get a superior orchid plant and varies in large quantities. The propagation starts from pollination to ripening of the resulting fruit and seeds and then grows in vitro. However, this generative propagation also has limitations where getting mature seeds takes a long time, at least 90 days after pollination (Yusnita and Handayani 2011). Only explants of moon orchids with a minimum age of 90 days after pollination can grow, and at that age, protocorm is produced in in-vitro culture (Mweetwa et al. 2008).

On the other hand, some research is on auxin's role in the orchid development process; moreover, endogenous auxin plays an important role in the development of orchids, from pollination to ovule development (Novak et al. 2014). The presence of auxin that accumulates after pollination causes continued development in the ovaries and ovules in orchid fruits. The auxin accumulation will encourage the ovaries and ovules to develop properly, so the fertilization process will also succeed in forming embryos.

Based on Novak et al. (2014) research, the failure to use orchid fruit explants under the age of 90 days after pollination other than because it does not have embryos can also be due to the dynamics of the auxin. Fruits harvested

early cause the distribution of auxin for the development of the ovaries and ovules to stop. Low levels of auxin in the immature orchid fruit explants can cause the failure of ovule development. Next, the failure of ovule development in in-vitro cultures causes the explants to be unable to grow.

The use of immature fruit explants as culture explants can provide many benefits. Immature fruits under 90 days after pollination used as explants can shorten the harvest time. In addition, if used as culture explants, immature fruits undergoing ovule development can be induced to produce haploid plants when fertilization has not occurred. Another advantage is that it is possible to produce uniform diploid plants that are not the result of the fusion of gametes. Based on the advantages of using immature orchid fruits, it is necessary to optimize their development to be used as in vitro culture explants.

Harvesting immature fruit 60 days after pollination causes the distribution of endogenous auxin to stop. It must be overcome because auxin plays an essential role in the development of the ovule. In this study, exogenous auxin was given to meet the needs of stopped endogenous auxin. This study aims to determine the effect of the exogenous application hormone auxin on tissue culture media on the induction and proliferation of protocorm in in-vitro cultures of immature moon orchid fruits.

MATERIALS AND METHODS

Plant material

The material needed is the moon orchid plant (*Phalaenopsis amabilis*) that has flowered. The moon orchid plant used comes from PT. Eka Karya Graha Flora and obtained at orchid nursery, Surakarta.

Procedure

This study used a Complete Randomized Design with variations in NAA concentration as a treatment on 1/2 MS medium. Immature orchid fruits used as explants were fruits 60 days after pollination. The treatment consisted of 4 levels (control, 1ppm NAA, 5 ppm NAA, 5ppm TIBA) with 5 replications. Next, the determination of auxin concentration was based on the results of Gurel and Gurel's research (1998); Kalimuthu et al. (2006); Ori et al. (2014); and Parmar and Pant (2015), and modifications were made through preliminary tests.

Pollination and orchid fruit collection

Orchid pollen is taken with a toothpick along with anther. The anther is removed, and pollen attached to the toothpick is inserted into the stigma cavity (pistil) (Parnata 2005). Orchid fruits aged 60 days after pollination were collected by cutting fruit stalks using sterile scalpels.

In vitro culture

Tools sterilization

Glass and dissection utensils were wrapped in straw paper, and culture bottles were covered with aluminum foil and sterilized by wet heating using an autoclave at 121ºC, a pressure of 1 atm for 1.5 hours.

In vitro *culture media*

Media without hormone treatment was made using an instant 1/2 MS (Murashige-Skoog) media in which 1 liter of media was made by dissolving 2,215 grams of MS, 30 grams of sucrose, and 8 grams in 1 liter of aquades. The media solution was also adjusted at a pH of 5.8. If the pH is less than 5.8 1N NaOH was added, and if it is more than 5.8 1N HCl was added. Media with hormone treatment were added with NAA and TIBA solution according to the concentration used. The media were then heated to boiling and poured into a culture bottle, covered with aluminum foil, then sterilized using an autoclave at 121º C, a pressure of 1 atm for 15 minutes.

Sterilization of materials

Orchid fruits used as explants were washed with running water for 5 minutes. The orchid fruit was then soaked in sterile material for 5 minutes of each ingredient while stirring. Sequentially, the orchid fruits were soaked in sterile materials containing soapy water, 5% Clorox, and 10% povidone-iodine. Each immersion in sterile material, orchid fruits were rinsed with sterile aquades. The material was sterilized in LAFC by soaking 70% alcohol for 5 minutes and rinsed with sterile aquades 3 times.

Explants planting

Explant planting was carried out inside the LAFC (Laminar Air Flow Cabinet), which had previously been sterilized with UV and LAFC spraying with 70% alcohol. Orchid fruits as explants were cut using scalpels and tweezers with a transverse position along the ± 0.5 cm - 1 cm. The pieces of explants were planted in culture bottles containing treatment media and then covered with aluminum foil and plastic wrap.

Explants maintenance

Explants planted in culture bottles were stored in the incubation room and placed on a culture rack. The culture rack was equipped with fluorescent lamps; the lamp was placed at a distance of 40-50 cm from the culture bottle with an intensity of 1,000-4,000 lux. The temperature in the culture incubation room was maintained in the range of 16-20ºC using the air conditioner.

Explants anatomy preparations

The explants were cut 1 cm long, and then fixation was done with an FAA solution consisting of formalin, glacial acetic acid, and 70% alcohol with a ratio of 5:5:90 for 24 hours. First, the explant pieces were washed with graded alcohol (70%, 80%, 90%, 95%, and absolute alcohol) and vacuum suctioned the air bubbles for ten minutes at each washing stage. Next, the preparations were dehydrated with a mixture of alcohol/xylol at a ratio of 3:1, 1:1, and 1:3, followed by absolute xylol twice with each stage for 10 minutes and followed by xylol/paraffin (1:9) for 24 hours. The preparations were then infiltrated with pure paraffin for 24 hours and then made blocks and attached o the holder. The preparation material in the block was then cut

with a sliding microtome; the resulting tape was attached to the glass of the object. Finally, preparations were observed using a fluorescence microscope.

Data observation

Observation of morphological data in the form of color changes of the explants was carried out every week for 21 days after planting. The color changes of the explants in browning or vitreous were observed through a stereomicroscope. The explants' development in protocorm formation was observed 42 days after planting. The protocorm morphology formed in each treatment was observed through a stereomicroscope. The protocorms diameter in each treatment was measured using the ImageJ application. The observed data were in the form of explants' anatomy before and after planting. Anatomical data before culture was obtained from the explants of the immature fruit *P. amabilis* aged 60 days after pollination, and anatomical data after culture was obtained from explants planted at 7 and 21 days after planting. The Protocorms that had formed 100 days after planting from each treatment were observed in their development and morphology.

Data analysis

The morphology of the explants, the development of the explants, the anatomy of the explants before and after planting, and the development of the protocorms were presented descriptively. The description is done by describing and comparing the results obtained between treatments. The results of protocorm measurements through the ImageJ application were quantitatively analyzed using One Way ANOVA, and further tests were conducted with a DMRT level of 5%.

RESULTS AND DISCUSSION

Changes in the color of *P. amabilis* immature fruit culture explants for 21 days after planting

Changes in the color of the explants were observed to determine the response of immature fruit culture growth to the administration of growing regulatory substances and their effects on the results of the development of subsequent ovule cultures. Figure 1 shows the changes in the color of the explants during the 21 days of planting. Changes in the color that occurs in the explants were indicated by the presence of browning and vitreous (transparent). Browning was found in the control treatment, adding 1 and 5 ppm of NAA. The explants with the addition of 5 ppm TIBA underwent vitreous. Browning that occurred in explants could be caused by cutting immature fruit explants. Such cuts resulted in the release of phenolic compounds as signaling molecules and induced the activity of the polyphenol oxidase (PPO) enzyme (Boeckx et al., 2015). PPO enzymes can be induced by activating pro-enzymes already present in previous explants (Haruta et al. 2001). The activity of these enzymes produced a brownish color that indicated the accumulation of phenolic compounds. The accumulated and oxidized phenolic components cause browning (Guardo et al., 2012).

Browning increased during the 21 days of planting on control treatment and NAA application. The cultured explants underwent gradual browning and showed increasingly obvious browning. It indicates prolonged PPO activity has an increasingly strong browning effect (Chuanjun et al., 2015). Browning on NAA treatment looked slightly stronger compared to control. Gunes (2000) reveals a link between the auxin and the activity of the peroxidase enzyme that produces phenolic compounds. The peroxidase enzyme can catalyze the oxidation activity of the IAA. The presence of endogenous auxin in the form of IAA that naturally exists in the ovule (Novak et al. 2014) is thought to affect the occurrence of browning. The addition of exogenous auxin causes the auxin accumulated in the explants to get higher and increases the activity of the enzyme peroxidase. Increased activity of peroxidase enzymes allowed the formation of high phenolic compounds and led to stronger browning.

The color change that occurred in applying 5 ppm TIBA (Figure 1.D) was in the form of vitreous, the color of the explants that became transparent. The color changes of the explants became transparent 21 days after planting, accompanied by the appearance of protocorms. Vitreous or hyperhydration in tissue cultures is caused by high air humidity, reduced transpiration, and excessive water uptake in tissues (Sowa 2012). Vitrification of explants can also occur due to disturbed polyamine metabolism and trigger lignin biosynthesis failure (Piqueres et al. 2002). Explants that undergo vitrification in in-vitro culture due to anti-auxin TIBA treatment also occurred in the study by Detrez et al. (1988). Research by Detrez et al. (1988) reports on research on petiole culture (leaf stalk) *Beta vulgaris* on IM culture media (containing 1 ppm TIBA), resulting in a high frequency of bud formation with explants that undergo vitrification. However, that study did not explain the mechanism of TIBA in inducing vitrification in explants.

Development of ovules from immature fruit cultures of *P. amabilis* at 42 days after planting

The development of ovules from immature fruit cultures of *P. amabilis* orchids can be seen from the formation of a globular structure called a protocorm in each treatment given. This observation was conducted to determine the effect of treatment on the protocorm that was successfully induced and proliferated. Figure 2 shows that the ovules in all treatments developed to form a protocorm. However, there were several variations in the protocorm produced in each explant. These variations include formation time, color, and size of the protocorm.

The time of the emergence of the protocorm varied in each treatment; at 5 ppm, TIBA treatment, Explants experienced the fastest protocorm formation 21 days after planting (Figure 1). Explants on control and application of 1 and 5 ppm NAA simultaneously formed a protocorm 42 days after planting. The color of the protocorm was also different in each treatment. At 42 days after planting (Figure 2), it could be seen that the 5 ppm TIBA treatment

had a light yellowish green protocorm with a fine hair structure on its surface. Protocorm in the control treatment and administration of 1 and 5 ppm NAA had a white color with a slight brownish color due to browning.

Size became a significant difference in the protocorms resulting from each treatment (Figure 3). The protocorm of the 5 ppm TIBA treatment had the widest circular area and had a significant difference compared to other treatments. Although the protocorm in the control treatment and 5 ppm NAA had the same size, the protocorm in the 1 ppm NAA treatment had the smallest size. There was no significant difference in the size of the protocorm produced in the control treatment and the administration of NAA. Table 1 shows that although the protocorm in the 1 ppm NAA treatment had the smallest protocorm size, the results did not significantly differ between the control treatment and 5 ppm NAA.

Table 1. The average protocorm size produced in each treatment

Treatments	42 DAP	
	Average protocrome diameter ± SD	Average protocrome area circumference ± SD
Kontrol	0.26[a] ± 0.03	0.05[a] ± 0.01
NAA 1 ppm	0.24[a] ± 0.06	0.04[a] ± 0.03
NAA 5 ppm	0.27[a] ± 0.05	0.05[a] ± 0.02
TIBA 5 ppm	0.32[b] ± 0.13	0.23[b] ± 0.09

Note: Numbers followed by the same letter do not show a significant difference according to the results of the DMRT follow-up test at the 5% level in the same column. SD: standard deviation

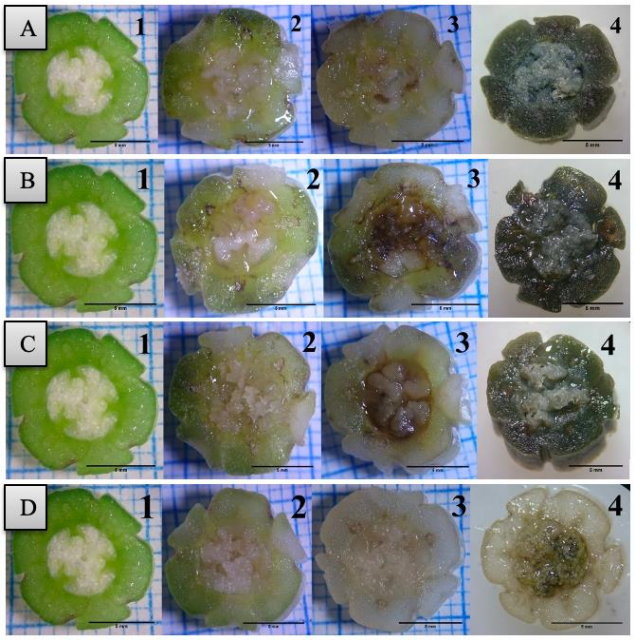

Figure 1. Changes in the color of the immature fruit culture of *P. amabilis* on various growth regulators treatments. A. control; B. 1 ppm NAA treatment; C. 5 ppm NAA treatment; D. 5 ppm TIBA treatment; 1. culture at 0 DAP (Days After Planting); 2. culture at 7 DAP; 3. culture at 14 DAP; 4. culture at 21 DAP

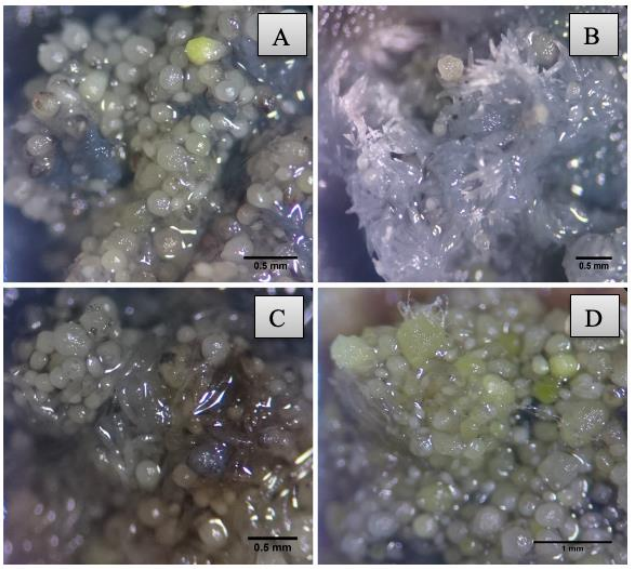

Figure 2. Development of ovules from immature fruit cultures of *P. amabilis* to form a protocrome at 42 DAP by observing through a 40X magnification stereo microscope. A.protocrome on control; B.protocrome at 1 ppm NAA treatment; C. protocrome at 5 ppm NAA treatment; D. protocrome at 5 ppm TIBA treatment

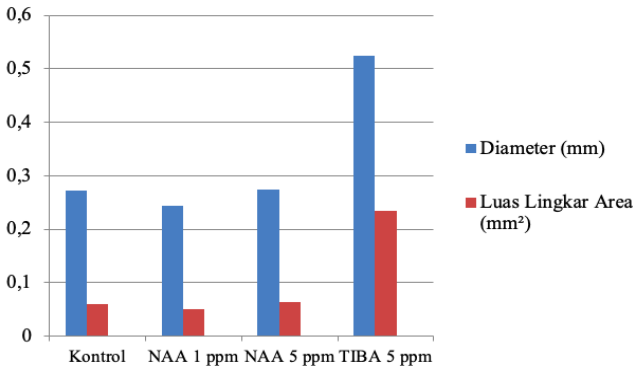

Figure 3. The average diameter and circumference of the protocorm area for each treatment

In the control treatment and the administration of 1 and 5 ppm NAA, each was able to form a protocorm simultaneously, the same size and brownish-white color. It indicates that the explants were able to produce protocorm under browning conditions. Furthermore, this is similar to the research results by Yamazaki and Miyosi (2006) regarding the germination of immature *Cephalanthera falcata* orchid seeds. Research by Yamazaki and Miyosi (2006) reports a higher frequency of germination in 60-80 days after pollination cultures experienced browning than in 90-120 days after pollination seed cultures that were not browning or slightly browning on culture media. Research on cultures that experienced browning was similar to that of Mii (1976), where the growth of explants was in line with the occurrence of browning in tobacco pollen cultures. Yamazaki and Miyosi (2006) suggest the need for further

experiments to evaluate whether browning in culture has a stimulatory effect on orchid germination.

The TIBA 5 ppm treatment in this study showed the ability of explants to produce protocorm with a faster time, yellowish-green color, and larger size than other treatments. However, this shows that the application of TIBA, which functions as an anti-auxin in the media, produces a protocorm with the best results from all treatments. The administration of TIBA did not inhibit the protocorm's formation but promoted the protocorm's induction and proliferation. It is similar to Chen and Chang's research (2004) regarding the positive response of TIBA to the in vitro culture of *Oncidium* orchid leaves. Chen and Chang (2004) report that were applying 0.5 µM TIBA on Oncidium leaf culture produced the highest average number of embryos.

Explants that produced a positive response to in vitro culture due to TIBA treatment also occurred in several studies. Tetu et al. (1990) report that was giving TIBA with a concentration of 0.2 µM increased shoot proliferation in the meristematic and cotyledon areas of *Pisum sativum* zygotic embryo culture. Sreenath et al. (1995) report that 1 and 5 ppm TIBA administration encouraged the fastest callus proliferation in coffee integumentary cultures. That is possible because the antagonist effect of TIBA can determine the balance of auxin and/or cytokinin for the growth of explants. The study also indicates that cells produce sufficient amounts of endogenous growth hormone without the exogenous addition of auxins or cytokinins.

This study showed that the administration of TIBA positively responded to the immature fruit culture of *P. amabilis*, which is possible that the application of TIBA is able to overcome the excessive level of auxin due to the production of endogenous auxin from explants in high quantities. TIBA is a specific IAA transport inhibitor (Sreenath et al. 1995). TIBA can interfere with IAA activity by competing for the same binding site as IAA, moving along the same path as IAA's polar transport, and having auxin competitor properties (Katekar and Geissler 1980). In addition, TIBA acts as an inhibitor of the carrier structure during auxin efflux inhibitor (Michniewicz et al. 2007). It is also possible that TIBA, an auxin competitor and auxin efflux inhibitor, can balance the need for auxin and/or other hormones in the cells. Hence, it provides a positive response to in vitro cultured explants. Further research is needed to determine how the TIBA mechanism can provide a positive response to tissue culture research.

P. amabilis ovule anatomy at 21 days after planting

Figure 4 shows the cross-sectional structure of the globular structure that was successfully induced and proliferated in each treatment. Anatomical data revealed that at 7 days after planting, the seed structure could develop into a globular structure at 21 days after planting for all treatments. The development of the seed structure to form a globular structure is characterized by the presence of a swollen embryo that is oval.

Observation results of the anatomy of *P. amabilis* ovules after 21 days of culture showed that the globular structure was successfully formed from the ovule structure 60 days after pollination was grown in vitro. The globular structure is characterized by the embryo's development in the integumentary structure. The development of the globular structure was similar to the structure of the seeds 99 days after pollination in *P. aphrodite* species. Jean et al. (2011) reported that the structure of *P. aphrodite* seeds aged 99 days after pollination showed the presence of seeds protected by seed coats with developing embryos.

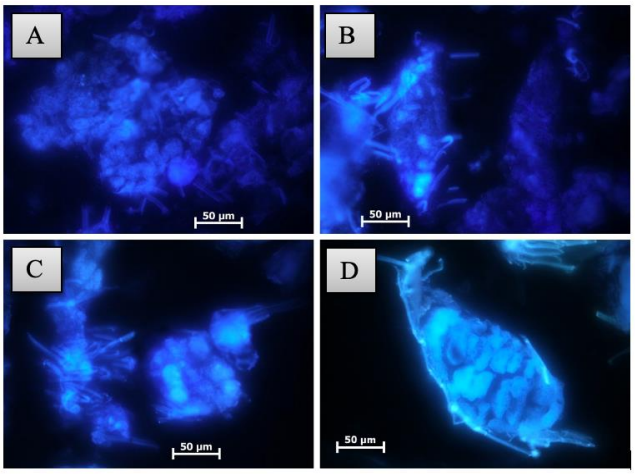

Figure 4. Cross-section of the globular structure of *P. amabilis* at 21 DAP by fluorescence microscopy. A. control treatment protocorm; B. protocorm of 1 ppm NAA treatment; C. protocorm of 5 ppm NAA treatment; D. protocorm of TIBA 5 ppm

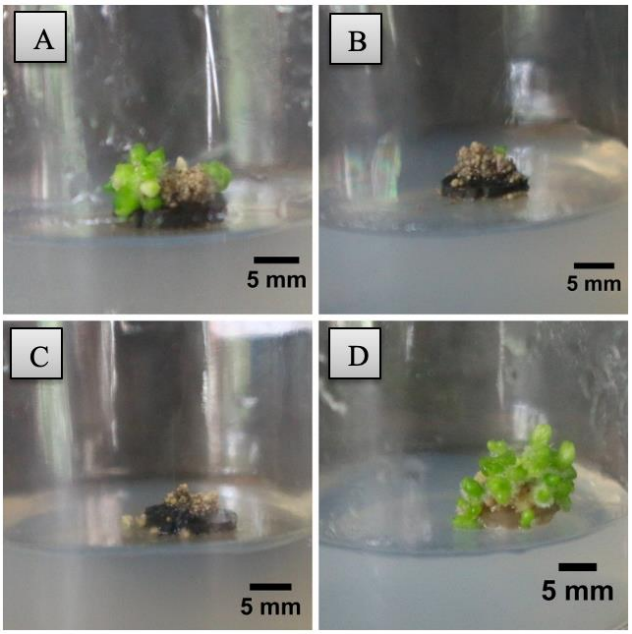

Figure 5. Protocorm development of *P. amabilis* immature fruit culture at 100 DAP. A. protocorm in the control treatment; B. protocorm of 1 ppm NAA treatment; C. protocorm of 5 ppm NAA treatment; D. protocorm of 5 ppm TIBA treatment

Development of protocorm *P. amabilis* at 100 days after planting

The protocorm that was induced and proliferated due to the development of the *P. amabilis* ovule was observed to continue its development. Furthermore, it was done to determine the effect of each given media treatment on the development of the protocorm. This observation was also carried out to determine the protocorm regeneration potential of *P. amabilis* immature fruit culture in in-vitro.

The protocol for the treatment with NAA (Figures 5.B and C) showed different results from the control treatment and the application of 5 ppm TIBA. The protocorm in the NAA treatment did not experience further development, and the protocorm formation stage seemed to have stopped. In the 5 ppm of NAA treatment, the growth of the explants showed a decline, and the protocorm produced was also less than in the 1 ppm NAA treatment. The protocorm in this treatment stopped at the formation of the protocorm, which was round and brownish white.

The treatment of giving NAA concentrations of 1 and 5 ppm in this study was considered toxic to the protocorm. The concentration of exogenous auxin and the level of endogenous auxin that is too high may cause failed embryogenesis (Sreenath et al. 1995). In addition, the phenolic compounds produced during culture can inhibit further protocorm development. Furthermore, this is similar to the research of Chen et al. (2015), in which the seeds of *Paphiopedilum spicerianum* grown on RE media (Robert Ernst medium) produced a protocorm that remained white, did not develop chlorophyll further and turned brown, and died. Moreover, ER media presence, which is toxic to some embryonic cells, is thought to affect the formation of chlorophyll.

Failure of protocorms development in NAA treatment may also be possible due to inappropriate exogenous auxin concentrations. Exogenous administration of auxin has varying results for each species, and explants are used where concentration is of utmost importance (Pradan et al., 2014). Therefore, the combination of concentration and ratio between auxin and cytokinin is very important. Some researchers found that low auxin concentrations can increase germination, and high auxin concentrations can inhibit orchid germination (Novak et al., 2014). The study showed that inappropriate hormone concentrations could inhibit the development and germination of orchid seeds.

The protocorm in the control treatment (Figure 5.A) showed a development in which some of the protocorms produced changed color from white to yellowish-green. In addition, some control treatment protocols could develop chlorophyll further and produce leaf primordia. The formation of meristems in orchid protocorms also requires an auxin organizing center that can regulate shoot development through polar auxin transport (Novak et al., 2014). Therefore, it can allow for the success of the control treatment protocorms, which can develop apical meristems due to sufficient endogenous auxin for protocorms development.

The 5 ppm TIBA treatment (Figure 5.D) showed that all the protocorms produced could change color from white to yellowish-green by showing the development of chlorophyll accompanied by the appearance of leaf primordia in the apical part. With the role of auxin efflux inhibitor from the addition of TIBA, the regulation of endogenous auxin transport in shoot development may become more balanced so that the protocorm can develop better.

This study found that the administration of 1 and 5 ppm NAA in the immature fruit culture of *P. amabilis* could only induce the formation of protocorms. Still, the protocorms were not able to develop further. The application of 5 ppm TIBA as an auxin transport inhibitor induced the formation of protocorms and developed all protocorms to form primordia leaves. Ovule culture, without the addition of hormones, induced the formation of protocorms and developed some of the protocorms to form primordia leaf. Further research is needed to obtain information on the ploidy of orchid plants produced from the culture of the immature fruit of *P. amabilis*, aged 60 days after pollination.

In conclusion, the immature fruit culture of the 60 days after pollination of *Phalaenopsis amabilis* orchid with the addition of exogenous auxin in the form of NAA with a concentration of 1 and 5 ppm was able to induce protocorms. Still, the protocorms were unable to develop further. Immature fruit cultures without the addition of hormones were able to induce protocorm and developed some protocorms to form primordial leaves. Finally, applying an auxin transport inhibitor in the form of TIBA with a concentration of 5 ppm induced the protocorms and developed all the protocorms to form primordial leaves.

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Effect of BAP and 2,4-D on callus induction of *Jatropha curcas* in vitro

SETIANINGRUM ANDARYANI, SAMANHUDI♥, AHMAD YUNUS
Department of Agronomy, Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia.
Tel.: +62-271-637457, ♥email: samanhudi@staff.uns.ac.id

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Abstract. *Andaryani S, Samanhudi, Yunus A. 2019. Effect of BAP and 2,4-D on callus induction of* Jatropha curcas *in vitro. Cell Biol Dev 3: 56-65.* The aim of this study was to obtain appropriate concentrations of Benzyl Amino Purine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) to increase callus induction in vitro using shoot explants of *Jatropha curcas* L. The research was conducted at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia, from July to August 2010. The study used a completely randomized design (CRD) with two treatment factors and three replications. The first factor is the level of BAP concentration, namely: 0.5 ppm, 1 ppm, 1.5 ppm, and 2 ppm. The second factor is the concentration of 2,4-D, namely: 0 ppm, 0.25 ppm, 0.5 ppm, and 0.75 ppm. Observation variables included callus emergence time, callus color, callus texture, root emergence time, number of roots, shoot emergence time, number of shoots, leaf emergence time, number of leaves, and callus fresh weight. Qualitative data are presented descriptively. Quantitative data includes callus fresh weight data, which was analyzed for variance based on the 5% F test and continued with the DMRT test at the 5% level. The results showed that the combination of 2 ppm BAP treatment and 0.25 ppm 2,4-D was the fastest in inducing callus, 5.67 Days After Planting (DAP). All the resulting calluses have a crumb texture. The average callus color in all treatment combinations was yellowish-green. Only the treatment combination of 1 ppm BAP without 2,4-D could produce roots at 9 DAP. The combination of 0.5 ppm BAP treatment and 0.25 ppm 2,4-D was the fastest to produce shoots, 6 DAP. The fastest leaf emergence was obtained in the 1 ppm BAP treatment without 2,4-D, 11 DAP. The combination of 2 ppm BAP treatment and 0.5 ppm 2,4-D resulted in the largest callus fresh weight (2.56 g).

Keywords: Benzyl Amino Purine, BAP, callus, dichlorophenoxyacetic acid, 2,4-D, in vitro, *Jatropha curcas*

INTRODUCTION

The need for fuel in Indonesia has recently been increasing along with the increase in oil-fueled transportation and other machines that use oil fuel. Now, Indonesia is no longer an exporter of petroleum but only an importer, especially from Arabia (Sumanto 2005). For this reason, it is necessary to find alternative sources and Jatropha (*Jatropha curcas* L.) is one of the plants that have the potential as a source of biofuel.

As a biofuel, Jatropha has several advantages compared to other plants. First, this plant has only a few limited functions, so competition for its use is also limited. In addition to being environmentally friendly, jatropha oil is not an edible oil, so the price of raw materials is lower, and it is not considered food (Prastowo 2007).

According to Syah (2006), the main problem in developing biodiesel from jatropha oil is the availability of raw materials, which is still very low, considering that the plantations have just been developed. Therefore, it is necessary to accelerate the productive Jatropha cultivation business to meet the raw material needs of the national biodiesel industry.

Along with the increasing demand and need for Jatropha plant material, it is necessary to make efforts to propagate plants in large quantities and in a short time. The provision of superior seeds is one of the factors supporting the success of Jatropha development. However, conventional propagation of plants is still limited by the ability of plants to produce new seeds in large quantities, uniformly, and in a short time. Until now, Jatropha seedlings have been produced in two ways: seeds and cuttings. However, the jatropha propagation business using cuttings or seeds has problems. Using seeds for plant propagation in large quantities will reduce the number of seeds processed into oil. In addition, the propagation technique through cuttings requires many parent trees. At the same time, the availability of parent trees is very limited, and there are also concerns about damage to the parent trees (Lizawati et al., 2009).

Cultivating tissue culture (in vitro) is necessary to overcome the problem above. Plant tissue culture is a technique for growing plant parts, either in the form of cells, tissues, or organs, under aseptic culture conditions in vitro (Yusnita 2004). Therefore, propagation by tissue culture will offer a great opportunity to produce large numbers of seeds in a relatively short time. In addition, tissue culture can maintain superior parental characteristics and produce seeds free of fungi, bacteria, viruses, and pests (Prihandana and Hendroko 2006).

The principle of this tissue culture technique is that all plant parts, whether in the form of cells, tissues, or organs, could become newer plants if grown under aseptic conditions in a sterile manner. Therefore, Jatropha tissue culture techniques will work well if the conditions are met. These techniques include the selection of explants as planting material, using a suitable medium, aseptic conditions, and good air regulation (Hendaryono and Wijayani 1994).

One of the factors influencing the success or failure of procuring Jatropha seedlings through tissue culture is the presence of growth regulators (ZPT). However, the hormone content in plants must also be considered. Hormones in plants are also called phytohormones. According to Pierik (1987), phytohormones are compounds produced by higher plants endogenously. These compounds play a role in stimulating and increasing the growth and development of plant cells, tissues, and organs towards a certain direction of differentiation. Other compounds with the same characteristics as hormones but are produced exogenously are known as PGR. Wetter and Constable (1991) suggested that one of the most frequently used compounds to induce cell division is 2,4-dichlorophenoxyacetic acid (2,4-D). In in vitro cultivation, callus induction is an important step. Suppose the endosperm of dicot plants is used, and hormones from the auxin group, namely 2,4-D or IAA, are added to the medium. In that case, hormones from the cytokinin group, namely kinetin or Benzyl Amino Purine (BAP), must be added (Suryowinoto 1996).

This study aimed to obtain the appropriate concentrations of BAP and 2,4-D to induce callus of Jatropha in vitro.

MATERIALS AND METHODS

Research material

The plant material used as explants was shoots of *J. curcas,* derived from sterile germinated seeds.

Research design

This study used a completely randomized design (CRD) arranged in a factorial manner with two treatment factors. The first factor was the concentration of BAP consisting of 4 levels, namely: B1 = treatment with the addition of 0.5 ppm BAP, B2 = treatment with the addition of BAP 1 ppm, B3 = treatment with the addition of 1.5 ppm BAP, B4 = treatment with the addition of 2 ppm BAP. The second factor is the concentration of 2,4-D consisting of 4 levels, namely: D1 = treatment without the addition of 2,4-D (0 ppm), D2 = treatment with the addition of 2,4-D 0.25 ppm, D3 = treatment with the addition of 2,4-D 0.5 ppm, D4 = treatment with the addition of 2,4-D 0.75 ppm. Thus, 16 treatment combinations were obtained, each repeated three times.

Research procedure

Tool sterilization

The equipment for this research were culture bottles, scalpels, petridish, and tweezers. First, they were washed using laundry soap, rinsed, then dried. Next, the drying equipment was wrapped in newspaper (except for culture bottles). Finally, all types of equipment were sterilized by autoclave at a temperature of 121°C and a pressure of 1.5 Psi (kg/cm^2) for 45 minutes.

Stock solution preparation

The stock solutions were prepared by weighing the chemicals, macronutrients, micronutrients, and PGR according to the composition of MS media (Appendix 1). These materials were dissolved with sterile distilled water and then stirred until completely homogeneous using a magnetic stirrer. The materials were then put into bottles, labeled according to the treatment, and stored in the refrigerator.

The making of planting media

The planting media was made by taking and measuring each stock solution according to the treatment and the determined size and then putting it into a measuring flask. These materials were dissolved with aquadest until the volume of the solution reached 250 mL (¼ liter) and added with 7.5 g sugar. After that, the solution was put in a glass beaker and stirred using a magnetic stirrer. The solution was conditioned at pH 6.3 by adding NaOH (if the pH was too low or HCl (if the pH was too high). Then, the solution was added with 2 g of agar, stirred with a magnetic stirrer, and boiled with a hot plate. After boiling, the solution is poured into culture bottles ± 25 mL each. The bottle was closed with 0.3 mm PP plastic and tied with rubber. The media was sterilized by autoclave at a temperature of 121°C at a pressure of 1.5 kg/cm^3 for 45 minutes. After that, the bottles were placed on the culture shelves.

Seed germination

Seed germination was carried out in a Laminar Air Flow Cabinet (LAFC), which had been previously cleaned with denatured alcohol. Before germination, the seeds were washed with soapy water and rinsed thoroughly. Then, their seed coats were peeled off with pliers.

The planting begins by bringing the mouth of the culture bottle close to the Bunsen lamp. During the planting, the mouth of the bottle must be close to the Bunsen lamp to prevent contamination. Next, the seeds were sterilized with 100% chlorox solution for 1 minute. Afterward, the seeds were opened, and the embryos were taken using tweezers and a scalpel. Then, they were planted on MS media without ZPT. First, the bottles were covered with aluminum foil, and then they were covered again with 0.3 mm PP plastic. Finally, the bottle with the embryo in it was labeled according to the planting date.

Explants planting

The explants used were the shoots of 11-day-old plants that had been germinated sterile. The explants were planted by taking the plant from the bottle with tweezers and then placing it on a petridish. The shoots of the plant were ready to be cut using a scalpel. First, the mouth of the bottle was preheated with a Bunsen lamp to prevent contamination. Then the explants were planted on the treatment media with sterile tweezers. The scalpel and tweezers were always heated to maintain the sterilization of the tool. Before closing, the mouth of the bottle was heated again. After that, the bottles were covered with aluminum foil and covered again with 0.3 mm PP plastic. Bottles were labeled according to the treatment and date of planting.

Maintenance

Maintenance of culture bottles was done by placing them on the culture shelves and spraying them with denatured alcohol every two days to prevent contamination.

Research variable

Observations were done daily by counting the days of the first callus appearance, expressed in days after planting (DAP). The callus appearance was indicated by swelling or the appearance of greenish-white tissue on the surface of the explant.

Visual observation of callus color was carried out at the end of observation (30 DAP). A score determined the callus color, i.e., 0: white, 1: whitish-green, 2: yellowish-green, 3: green, 4: brownish-green.

Callus texture was observed at the end of the observation (30 DAP) by observing the formed callus texture, whether compact or crumbly.

The time of emergence of the first roots was calculated from the time of planting until the emergence of roots and expressed in DAP. It was characterized by the presence of yellowish-white protrusion (± 2 mm) on the lower part of the explant.

The number of roots was observed by counting the total roots in each growing explant. It was done at the end of the observation (30 DAT).

The first shoots' emergence time was calculated from planting until the emergence of shoots and expressed in DAP. The formation of shoots was indicated by the presence of a greenish-white protrusion (± 2 mm) on the upper part of the explant.

The number of shoots was observed at the end of the observation (30 DAP) by counting the number of shoots that emerged from the surface of the explants.

The observation at the time of leaf emergence was carried out using the same research method as Nofiyanti's in 2007, namely by counting the days from planting until the opening of the leaves completely. It was expressed in DAP.

The number of leaves was observed by counting the entire leaves in each growing explant. It was performed at the end of the observation (30 DAP).

Callus Fresh Weight was observed at the end of the observation (30 DAP). The fresh weight of callus was measured by weighing on an analytical balance the fresh weight of callus along with culture bottles complete with lids minus the weight of culture bottles (without callus) and lids.

$$WW = WW_t - WW_O$$

Note:
WW : fresh callus weight (g)
WWt: fresh callus weight+culture bottle+cover (g)
WWo: weight of culture bottle+cover (g)

Data analysis

The qualitative analysis included visual data. In addition, the data were analyzed using descriptive methods. Meanwhile, quantitative data were analyzed using analysis of variance based on the F test at a 5% level. If there was a significant difference, it was continued with the DMRT test at a 5% level.

RESULTS AND DISCUSSION

Callus appearance time

One indicator of the growth of in vitro culture is the appearance of callus on explants. According to Hendaryono and Wijayani (1994), callus is undifferentiated cells formed on one or all of the explant slices. In this study, the callus was first formed at the tip of the explant in contact with the media. Next, start with swelling of the explants, then callus appears on the base of the explants with a greenish color, according to Hartmann et al. (1990) cit. Dwiyono (2009), callus produced through in vitro propagation is formed due to tissue injury and hormone response. The average callus appearance of Jatropha explants at various concentrations of BAP and 2,4-D is presented in Table 1.

Table 1 shows that callus was induced in almost all treatments. Auxins are generally added to the nutrient media to induce callus from explants (George and Sherrington 1984). In this study, callus was not formed in explants that were not added with 2,4-D. The absence of callus was possible because endogenous auxin in Jatropha explants had not been able to induce callus. In other words, explants had low auxin content, so they still needed additional exogenous auxin in the culture medium. Pierik (1987) stated that auxin is a hormone capable of inducing callus. It is reinforced by Haensch's (2007) statement that the combination without 2,4-D does not show any somatic embryogenesis. Somatic embryogenesis is the process of the formation of embryos derived from somatic cells (not the result of the fusion of male and female gametes). Explants often die or do not change. However, some of the explants formed a little callus.

This study showed that the B4D2 treatment (BAP 2 ppm and 2,4-D 0.25 ppm) was the fastest to induce callus at 5.67 DAP. It is in line with Hanifah's research (2007), in the treatment of 0.5 ppm NAA and 1 ppm BAP and 0.5 ppm and 2 ppm NAA treatment gave the fastest callus appearance of 13.33 DAP. Although auxin is known as a hormone capable of inducing callus, cytokinins are often used as a combination ingredient for callus induction.

Gustian (2009) states that adding auxin at low concentrations will generally stimulate callus formation. On the contrary, if the ratio of auxin and cytokinin in the medium is higher, it will stimulate callus explants to regenerate to form organs. In addition, Table 1 shows that the longest callus induction was obtained in the B1D4 treatment (BAP 0.5 ppm and 2,4-D 0.75 ppm) at 7 DAP. It was possible because the concentration of 2,4-D given to the explants was high, thus inhibiting callus growth in the explants. At high levels, auxin inhibits rather than stimulates growth (Hendaryono and Wijayani 1994). The inhibition of callus growth was due to the culture mass being grown for too long in the same medium, which caused the loss of nutrients and water. Running out of nutrients and water can occur because, apart from being

sucked in for growth, the media evaporates water from time to time. In addition to running out of nutrients, the callus releases compounds resulting from metabolism, eventually inhibiting the callus's growth (Anonim 2010).

Callus color

Explant growth indicators of *in vitro* cultivation in the form of callus color describe the visual appearance of the callus so that it can be known whether a callus still has cells that are actively dividing or have died. Callus tissue produced from an explant usually shows different colors. Good quality callus has a green color. According to Fatmawati (2008), callus color indicates the presence of chlorophyll in the tissue. The greener color of the callus means more chlorophyll content. A light or white color can indicate that the callus condition is still quite good.

Figure 1 showed that the color in the callus of J. curcas ranged from white (indicated by a score of 0) to brownish-green (indicated by a score of 4). Based on Table 2, the color of the callus ranges from whitish-green, yellowish-green, and brownish-green. The difference in callus color indicated that the level of callus development was different. Almost all treatments showed a yellowish-green color on the formed callus. According to Hanifah (2007), adding cytokinins with increasing concentrations tends to show a green (bright) color on the callus to last longer. The green color of callus is due to the effect of cytokinins in the formation of chlorophyll. In contrast, the whitish-green callus color was shown in the BAP treatment of 1 ppm and 2,4-D 0.25 ppm.

Brownish green callus color was found in the treatment of 2,4-D 0.75 ppm, which was added with 0.5 ppm BAP and 1 ppm BAP. Callus color getting darker (becomes brown) means that callus growth is decreasing. In this study, callus with a brownish-green color was produced on media containing 2,4-D with a fairly high concentration. The results of research reinforced by Dwiyono (2009) that the increasing addition of 2,4-D can lead to an increase in the formation of callus with brown color on *mahkota dewa* (*Phaleria macrocarpa* (Scheff.) Boerl.).

The brown color of the callus is due to the metabolism of toxic phenolic compounds, often stimulated by the explant sterilization process, which inhibits growth or even causes tissue death (Yusnita 2004). Santoso and Nursandi (2004) stated that the browning event was a natural event and a process of adaptive changes in plant parts due to physical influences such as stripping and cutting. Browning symptoms were signs of physiological deterioration of explants. In addition to indicating the occurrence of synthesis of phenolic compounds, the brown color was caused by the increasing age of cells or callus tissue. Following Palupi et al. (2004) research, brown callus is a callus that undergoes the cell aging process (senescence) due to the absence of BA in the media. Thereby this condition accelerates the aging process (senescence) of cells. High concentrations of 2,4-D and BA (2,4-D 1.0 mg/l and BA 1.0 mg/l) can stimulate the aging process, inhibiting the callus growth process.

Callus texture

Callus texture is a marker used to assess a callus's quality. A good callus is assumed to have a crumbly texture (friable). Crumbly callus texture is considered good because it facilitates the separation into single cells in suspension culture and increases oxygen aeration between cells. Thus, efforts to propagate the number of callus through suspension culture are easier with this texture. Callus texture can be divided into three types, namely: compact (non-friable), intermediate and crumbly (friable) (Turhan 2004). Visually, the bonds between cells in the crumb callus formed on the explants of *J. curcas* looked loose and easily separated. If taken with tweezers, the callus was easily broken, and some stuck to the tweezers.

On the other hand, a compact callus has a texture that is difficult to separate and seems solid (Fitriani 2008). Meanwhile, the callus, partly compact and crumbly, is called the intermediate callus (Widiarso 2010). Callus textures formed on Jatropha explants at various concentrations of BAP and 2,4-D are presented in Table 3.

Table 1. Callus appearance time in Jatropha explants at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	6	6	7
1	~	6	6	6
1.5	~	6	6.33	6.33
2	~	5.67	6	6.33

Note: ~= no appearance of callus, DAP= days after planting, ppm = part per million (mg/L)

Table 2. Callus color of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	yellowish-green	yellowish-green	brownish-green
1	~	whitish-green	yellowish-green	brownish-green
1.5	~	yellowish-green	yellowish-green	yellowish-green
2	~	yellowish-green	yellowish-green	yellowish-green

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

Table 3. Callus texture of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP (ppm)	2,4-D (ppm)			
	0	**025**	**05**	**075**
05	~	crumbly	crumbly	crumbly
1	~	crumbly	crumbly	crumbly
15	~	crumbly	crumbly	crumbly
2	~	crumbly	crumbly	crumbly

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

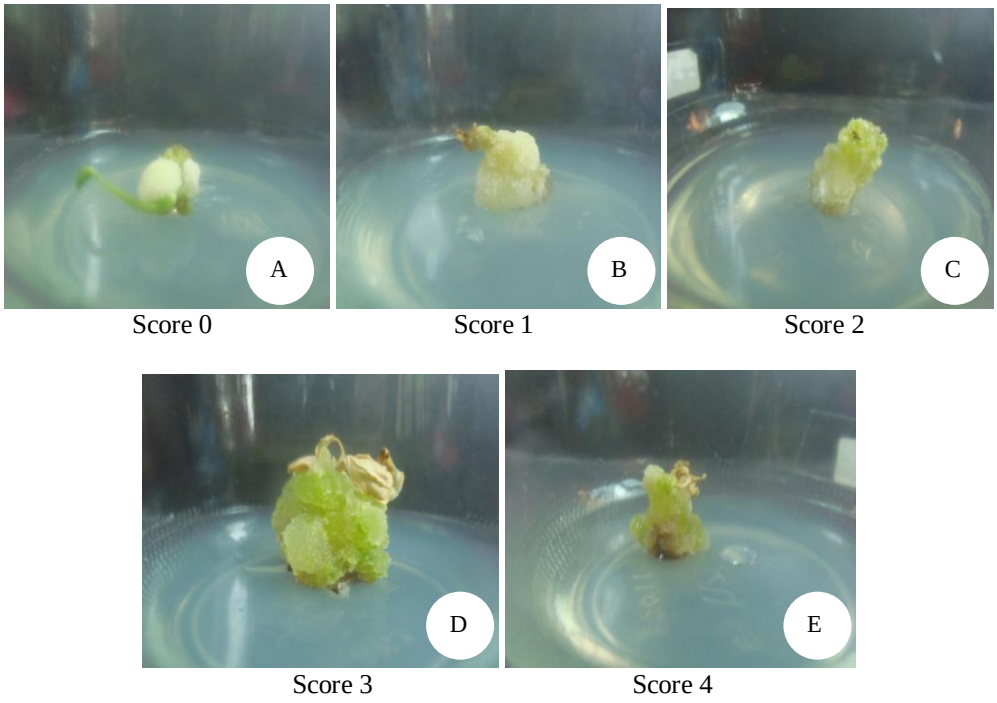

Figure 1. Callus color scoring category on Jatropha explants (A) white callus, (B) whitish-green callus, (C) yellowish-green callus, (D) green callus, (E) brownish-green callus

Pierik (1987) stated that the texture of callus could vary from compact to crumbly, depending on the type of plant, nutrient composition of the media, growth regulators, and environmental conditions of the culture. Table 3 showed that the combination of treatments formed callus with a crumbly texture, except for the treatment without 2,4-D. According to Fatmawati (2008), the callus that mostly had crumbly texture on leaf explants of *A. annua* was caused by 2,4-D in culture media. The same thing was also obtained in Ratnadewi's (1991) cit Fatmawati's (2008) research: Auxin 2,4-D combined with kinetin 1 mg/L was used to induce the formation and propagation of friable/crumbly callus in sugarcane plants. The formation of callus with a crumbly texture, according to Widyawati (2010), is triggered by the presence of endogenous auxin hormones produced internally by explants that have grown to form the callus.

Root appearance time

Roots for plant growth play a very important role because the roots are directly in contact with the planting medium where nutrients are stored. Zulkarnain (2009) said that roots function as a tool to absorb nutrients and nutrients and support the plant body. In addition, roots also function as transporters and food storage places such as carrots, sugar beets, and sweet potatoes. Therefore, the presence of roots is needed by plants, and in vegetative propagation, including tissue culture, various efforts are made to form roots. The emergence of roots in Jatropha explants at various concentrations of BAP and 2,4-D in vitro is presented in Table 4.

Table 4. The time of root appearance in Jatropha explants at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP (ppm)	2,4-D (ppm)			
	0	**0.25**	**0.5**	**0.75**
0.5	~	~	~	~
1	9	~	~	~
1.5	~	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, ppm = part per million (mg/L)

Table 4 shows that the BAP treatment of 1 ppm without adding 2,4-D could produce roots. In this study, root formation only occurred in the combination treatment B2D1 (BAP 1 ppm, 2,4-D 0 ppm) at 9 DAP. Most of the explants were unable to grow roots. It is suspected that with the addition of 2,4-D to the culture medium, the explants focused more on callus induction than root emergence. In addition, this may occur because the concentration of auxin (2,4-D) given in the medium is too little. Judging from the combination of treatments in this study, the ratio of auxin and cytokinin concentrations was low, so the treatment could not produce roots. It is in line with the statement of Yunus et al. (2007), which states that if the ratio of the concentration of auxin to cytokinin is low, then the plants with this treatment will not be able to grow roots. Therefore, it can be concluded that the treatment has the roots grow with a high ratio of auxin and cytokinin, including IBA 0 ppm and BA 3 ppm, IBA 0.25 ppm and BA 1 ppm, and IBA 0.5 ppm and BA 1 ppm. This condition deviates from the opinion of Wetherell (1982) that for root formation, it is necessary to have a low ratio of auxin and cytokinin. This deviation was maybe because the endogenous growth substances (endogenous auxin)

contained in the explants in the treatment were sufficiently available.

In organogenesis, three possibilities can cause explants to fail. First, cells in explants lack totipotency, that is, the total genetic potential, which means that every living plant cell is equipped with a complete genetic and physiological device to grow into a whole plant under the right condition. Second, cells in explants could not differentiate and dedifferentiate due to a lack of essential induction stimulation of any type or inappropriate concentration of growth regulators (Tripepi 1997 cit. Prihatmanti and Mattjik 2004).

Number of roots

Many roots can optimize the absorption of nutrients in the culture media. Nickell (1982) cit. Rahmaniar (2007) stated that the active auxins used for root formation were Naphthalene acetic acid (NAA) and Indole Butyric Acid (IBA). Some other types that can be used are 2,4-D and 2,4,5-T. Both types form roots when used at low concentrations. The type of root system produced also depends on the growth regulator used. For example, the phenoxy acids at 2,4-D and 2,4,5-T produce a rich, thick, sturdy root system. In comparison, IBA produces a strong fibrous root system.

In this study, only the combination treatment without 2,4-D with the addition of 1 ppm BAP was able to grow roots. The root produced in this study was only 1 single root. In stem explants, roots do not always appear at the nodes (Rahmaniar 2007) but can also appear at the stem base in the media (Figure 2). Visually, the roots formed on the explants of Jatropha shoots were yellowish-white without root hairs, thin, and not sturdy, as Rahmaniar (2007) stated that in the treatment without 2,4-D, the roots formed were single roots that were thin and not sturdy.

The cells in the explants are thought to be able to produce their auxin to encourage cell metabolic processes. Therefore, adding auxin to the culture media will cause an unbalanced interaction with endogenous auxin and cannot produce a larger number of roots (Mujiyanto 2003 cit. Rahmaniar 2007).

Shoot appearance time

Shoots are plant parts obtained from vegetative propagation, which grow to carry out offspring on the plant. The formation of shoots indicates the success of the regeneration of explants inoculated on tissue culture media. Callus resulting from callus induction of Jatropha explants can differentiate to form shoots. However, in this study, not all callus formed could differentiate into shoots, so several shoots formed directly. The faster the shoots appear, the faster the material for plant propagation will be produced. The average shoots of Jatropha explants at various concentrations of BAP and 2,4-D are presented in Table 5.

Table 5 shows that the average fastest shoot appearance of Jatropha explants was in the treatment of 0.5 ppm BAP with the addition of 2,4-D 0.25 ppm, namely 6 DAP. This treatment produced bud shoots. From Table 5, it can be concluded that the effect of BAP and 2,4-D gave different responses to the appearance of shoots, as stated by George and Sherrington (1984), that the initiation of shoots and roots is determined by the concentration of cytokinins and auxins given to the medium and their interactions with cytokinins or endogenous auxins contained by explants.

In the combination treatment of 0.5 ppm BAP, and 2,4-D 0.75 ppm, the slowest effect in stimulating the appearance of shoots was at 22 DAP. Meanwhile, Jatropha explants at 1 ppm, 1.5 ppm, and 2 ppm BAP treatment added with 2,4-D 0.75 ppm did not produce shoots. It means that adding 2,4-D to the explants could not accelerate the appearance of shoots. However, as Hariyanti et al. (2004) stated, the increase in exogenous auxin and inhibition effect on the time of shoot formation is also increasing. Therefore, jatropha explants with BAP treatment of 1 ppm, 1.5 ppm, and 2 ppm without 2,4-D were able to produce shoots. This statement is supported by Nursetiadi (2008) that the endogenous auxin found in explants has been able to encourage the formation of shoots, so it only requires auxin that is not too high.

Adventitious shoots also appear on some explants, forming a callus. Adventitious shoots are derived from explant cells or tissues that previously did not have buds (Yusnita 2004). At the end of the observation, green protrusions (±2 mm) were seen on the formed callus (Figure 3A). These protrusions are adventitious shoots that will grow into new shoots. Adventitious shoots appeared in the combination treatment of 0.5 ppm BAP and 2,4-D 0.75 ppm, 2 ppm BAP and 2,4-D 0.25 ppm, 1 ppm BAP and 2,4-D 0.5 ppm, and BAP 2 ppm and 2,4-D 0,5 ppm. The formation of adventitious shoots is thought to influence the addition of cytokinins, in this case, BAP, into the culture medium. It follows the opinion of Yusnita (2004), which states that cytokinins can stimulate the formation of adventitious shoots. However, George and Sherrington (1984) stated that the interaction between auxins and cytokinins influenced the formation of adventitious shoots.

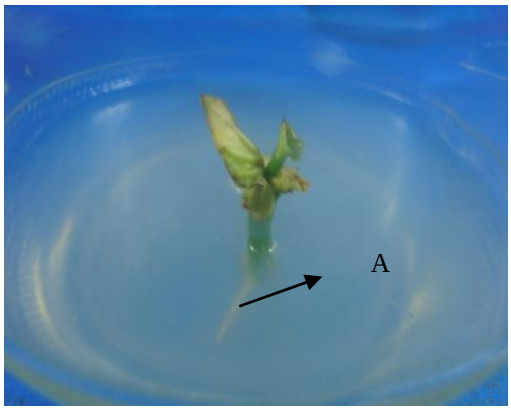

Figure 2. A root that was formed on jatropha explants in combination treatment B2D1 (BAP 1 ppm and 2,4-D 0 ppm)

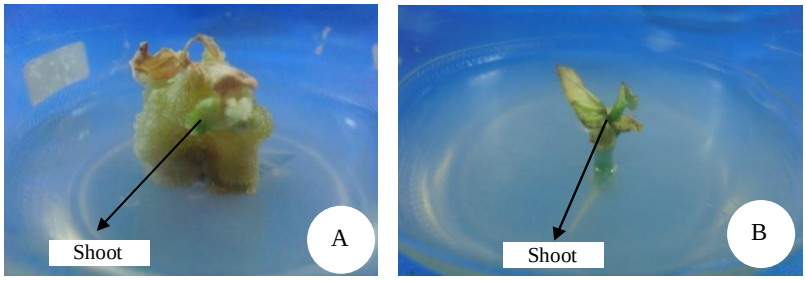

Figure 3. Types of shoots formed in Jatropha explants (A) adventitious shoots, (B) bud shoots

Hariyanti et al. (2004) reported that the higher the administration of exogenous auxin, the effect of its inhibition on the time of shoot formation also increased. However, in this study, with increasing concentration of 2,4-D, the effect of inhibition on the emergence of shoots was varied (Table 5); possibly, the explants contained endogenous auxin whose levels were not the same. Uniformity of size and method of taking explants is most likely not followed by the uniformity of endogenous plant hormones. Therefore, adding exogenous auxin into the culture media will cause a varied response.

Number of shoots

The number of shoots is the most important factor in plant multiplication in tissue culture. In tissue culture, the number of shoots can be indicated as success in multiplication. As many shoots are formed, multiplication of cultures to get more new shoots can be done. The number of shoots was calculated on all shoots that appeared on explants, both from elongation and adventitious shoots (not from shoots).

Figure 4 shows that the average shoot emergence in all treatments producing shoots was 1. The various concentrations of BAP and 2,4-D given in this study could not grow more than one shoot. The shoots formed came from the elongation of the shoots of the plant stems and shoots from the differentiation of callus tissue in explants. Therefore, it is in line with Nursetiadi's (2008) research which states that the types of media and the concentrations of BAP given in this study could not grow more than one shoot. Adding 1 to 3 ppm BAP concentrations tended to give the same results. Although the BAP concentration level had increased, there was no change; possibly that the administration of cytokinins with BAP concentrations of 3 ppm had not been able to stimulate shoot multiplication. According to George and Sherrington (1984), a higher cytokinin concentration than auxin concentration will stimulate shoot multiplication, which is reinforced by the statement of Haensch (2007) that the combination of 0 ppm 2,4-D with 4 ppm BAP causes shoot regeneration.

The role of auxin is to stimulate the division and enlargement of cells found in plant shoots and cause the growth of new shoots. Therefore, the addition of a larger amount of auxin, or the addition of a more stable auxin, such as 2,4-D acid, tends to cause callus growth from explants and inhibit plant shoot regeneration (Wetherell 1982).

Time of leaves appearance

Leaves are vegetative organs whose growth is influenced by the nitrogen content in the media. According to Pierik (1987), the source of organic N in tissue culture media is NH4+ and NO3-, and MS base media is the highest among other basic media. Therefore, using MS media can stimulate the growth of vegetative organs. However, almost all treatment combinations did not produce leaves. The average leaf appearance of Jatropha explants at various concentrations of BAP and 2,4-D is presented in Table 6.

Table 6 shows that leaves only appeared at 1 ppm BAP and 1.5 ppm BAP without adding 2,4-D and appeared at 11 DAP and 14 DAP, respectively. The factor that caused the absence of leaves in all treatment combinations was thought to be the explants dividing cells by dedifferentiation due to the addition of 2,4-D. As Yusnita (2004) stated, plant cells at the callus induction stage will experience dedifferentiation, namely the process of changing explant cells that were previously specialized to form plant organs such as roots and leaves or shoots to become no longer specialized. Under these conditions, the cells will return to being meristematic. Wetter and Constabel (1991) also said that cells growing on callus would be meristematic and usually undifferentiated.

Number of leaves

Leaves are the center of photosynthesis, a source of food for plants. So the more leaves, the better the plant growth is. The number of leaves is affected by adding growth regulators into the media. In this study, the average number of leaves that grew in the combination treatment without 2,4-D with 1 ppm BAP and 1.5 ppm BAP were 2 leaves and 1 leaf, respectively (Table 7).

Cytokinin hormones also influence leaf formation in tissue culture. In line with the statement of Yelnitis (1996) cit. Purwanto (2008) that adding BAP group cytokinins at a higher cytokinin ratio than auxin in the media can encourage an increase in the number of leaves. Widyawati's research (2010) also showed that only one treatment could form leaves, namely the 0.5 ppm BAP treatment without NAA. However, as in Hanifah's research (2007), treatment without NAA by giving 1 ppm BAP can produce the most leaves, namely 6 leaves. Therefore, it is suspected that adding cytokinins (BAP) in the media can encourage meristem cells in explants to divide and influence other cells to develop into shoots and form leaves.

Table 5. The appearance of Jatropha explant shoots at various concentrations of BAP and 2,4-D in vitro (DAP)

BAP	**2,4-D (ppm)**			
(ppm)	**0**	**0,25**	**0,5**	**0,75**
0,5	~	6	8	22
1	11	7	18	~
1,5	10	~ 14	~ 10,5	~
2	8			~

Note: ~ = no appearance of callus, ppm = part per million (mg/l)

Table 6. Time of emergence of Jatropha explant leaves at various concentrations of BAP and 2,4-D in vitro (HST)

BAP	2,4-D (ppm)			
(ppm)	0	0.25	0.5	0.75
0.5	~	~	~	~
1	11	~	~	~
1.5	14	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, DAP= day after planting, ppm = part per million (mg/l)

Table 7. Number of leaves of Jatropha explants at various concentrations of BAP and 2,4-D in vitro

BAP	2,4-D (ppm)			
(ppm)	0	0.25	0.5	0.75
0.5	~	~	~	~
1	2	~	~	~
1.5	1	~	~	~
2	~	~	~	~

Note: ~ = no appearance of callus, DAP= day after planting, ppm = part per million (mg/l)

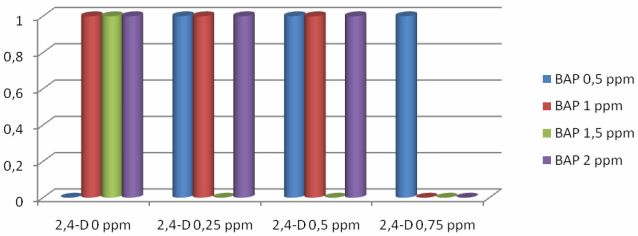

Figure 4. Histogram of the average number of *Jatropha* shoots with various concentrations of BAP and 2,4-D

In this study, some leaves changed color to brown, and some fell off; the leaves fell from the petiole base (Figure 5). It may be because the existing nutrients are not sufficient for the survival needs of the leaves until the explants can grow perfectly. It was suspected that the absorption was not optimal because there was no root formation in the explants; moreover, according to Supriyanto et al. (1992) cit. Triatminingsih et al. (1995), leaves that are formed experience loss due to chlorosis. Chlorosis is an event that decreases or decreases chlorophyll due to the addition of auxin. A combination of endogenous and exogenous auxin occurs in the tissue, then synthesizes ethylene which will cause leaf aging.

Callus fresh weight

An irreversible increase in weight characterizes growth. Therefore, the fresh weight measurement of the callus can represent the callus growth variable derived from shoots of Jatropha plant explants. According to Ruswaningsih (2007), physiologically fresh weight consists of two ingredients: water and carbohydrates.

The analysis of variance in the 5% F test showed that BAP and the interaction between BAP and 2,4-D had no significant effect. Meanwhile, the administration of 2,4-D in the media significantly affected the fresh callus weight (Appendix 16). The 5% DMRT test explained that the three treatments of 2,4-D (0.25, 0.5, and 0.75 ppm) had a different effect from the control (0 ppm 2,4-D) in increasing callus fresh weight (Table 8). In this study, the treatment without 2,4-D could not form a callus. It may occur because the endogenous auxin content in the explants was not sufficient to form a callus, so it still required exogenous growth regulators to form a callus. According to Santoso and Nursandi (2004), the direction of culture development is determined by the interaction and balance between growth regulators produced by plant cells endogenously because, in the explants, there are endogenous growth regulators. Still, in plant growth and development internally, the growth and development of plants are internal; therefore, in in-vitro exogenous growth, regulators are still being added. The addition of 2,4-D acid was carried out because 2,4-D acid played a role in promoting callus morphogenesis and callus induction and could affect the genetic stability of plant cells.

Based on the histogram (Figure 6), the highest callus fresh weight was 2.56 grams, obtained in the B4D3 treatment (BAP 2 ppm; 2,4-D 0.5 ppm) which was not much different from the fresh callus weight produced by B4D2 treatment (BAP 2). ppm; 2,4-D 0.25 ppm) of 2.48 grams. The callus formed in these two treatments was influenced by the presence of both endogenous and exogenous auxin with the addition of 2,4-D.

Rahayu et al. (2003) stated that the large fresh weight of callus was due to its high water content. The resulting wet weight is highly dependent on the speed at which these cells divide, multiply and continue with the enlargement of the callus. In addition, Pierik (1987) added that callus growth in one plant species might differ depending on the plant's original explant position and growing conditions. Growth and morphogenesis in in-vitro are influenced by interactions and balances between growth regulators added to the medium and growth hormones produced endogenously by cultured cells (George and Sherrington 1984). Therefore, BAP and 2,4-D treatments had no significant effect (ns) on fresh callus weight (Fatmawati 2008). Therefore, it could be concluded that the administration of 2,4-D 0.5 ppm was the best for fresh callus weight gain. However, giving 2,4-D 0.25 ppm was more beneficial because, besides having a very significant effect, adding a small concentration of it could increase the fresh weight of Jatropha callus.

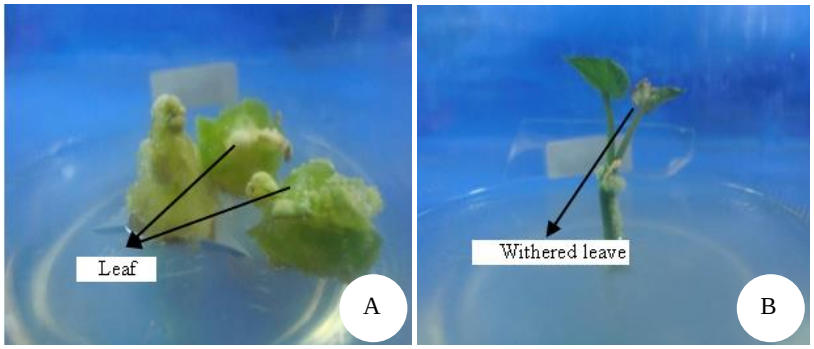

Figure 5. Jatropha shoots explants (A) Greenish yellow leaves fall off, which form a callus (B) Leaves turn brown (wither)

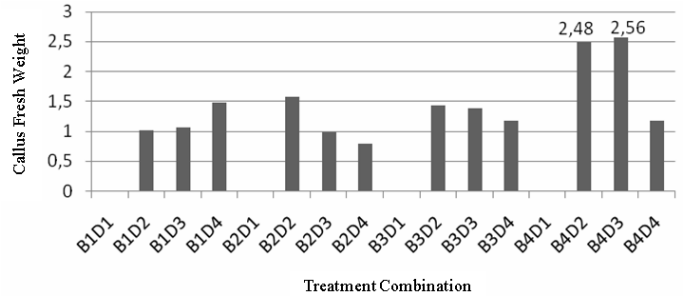

Figure 6. Effect of BAP and 2,4-D treatment on fresh weight of Jatropha callus in vitro. Note: B1: BAP 0.5 ppm; B2: BAP 1 ppm; B3: BAP 1.5 ppm; B4: BAP 2 ppm; D1: 2,4-D 0 ppm; D2: 2,4-D 0.25 ppm; D3: 2,4-D 0.5 ppm; D4:2,4-D 0.75 ppm

Table 8. Average fresh weight of Jatropha callus at various concentrations of 2,4-D in vitro

2,4-D (ppm)	Average callus weight
0	0.00 a
0.25	1.62 b
0.5	1.50 b
0.75	1.15 b

Note: Numbers followed by the same letter show no significant difference in the 5% DMRT test

In conclusion, concentrations of BAP 2 ppm and 2,4-D 0.25 ppm were the most optimal to induce the callus of *J. curcas* plant in vitro. The callus produced is generally yellowish-green in color and has a crumb texture. Adding 2 ppm BAP and 0.5 ppm 2,4-D produced the highest callus fresh weight of 2.56 grams. The combination of 1 ppm BAP treatment without 2,4-D produced roots at 9 DAP with 1 single root. Administration of 0.5 ppm BAP with the addition of 2,4-D 0.25 ppm gave the fastest shoot emergence, 6 DAP. The average of the fastest leaf emergence in BAP treatment was 1 ppm without 2,4-D, was 11 DAP with 2 leaves.

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Physical characteristics of the seeds of soybean (*Glycine max*) varieties and the effect of fermentation time on the chemical characteristics of tempeh

PRATIWI DWI SUHARTANTI, SRI HANDAJANI, NANDARIYAH♥
Department of Agronomy, Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia.
Tel./fax.: +62-271-637457, ♥email: nandariyah@staff.uns.ac.id

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Abstract. *Suhartanti PD, Handajani S, Nandariyah. 2019. Physical characteristics of the seeds of soybean (*Glycine max*) varieties and the effect of fermentation time on the chemical characteristics of tempeh. Cell Biol Dev 3: 63-68.* Soybean is the main raw material for making tempeh. So far, the raw material for tempeh is imported soybeans. Therefore, Indonesia needs to develop soybean varieties to overcome this. The soybean varieties are expected to be processed into tempeh with good physical and chemical properties. This study aims to determine the effect of different varieties on the physical characteristics of soybean seeds and the effect of fermentation time on the chemical characteristics of tempeh. The research design was a completely randomized design (CRD) with a factorial pattern consisting of two factors, namely soybean varieties (Grobogan, Argomulyo, Seulawah, Anjasmoro, Burangrang, and Galunggung) and variations in fermentation time (30, 42, and 54 hours). The results showed that the difference in varieties did not affect the color of the tempeh. Different varieties affect seed weight, water absorption, and swelling power of soybeans. Soybean varieties that have the best physical characteristics (highest water absorption and swelling power) are Grobogan. Variations in fermentation time affect the chemical properties of tempeh. Longer fermentation time will increase the tempeh's water, ash, and total protein content but decrease the fat and carbohydrate content. The soybean variety with the best chemical characteristics (highest protein content) is Galunggung. The soybean seed coat is yellow and greenish-yellow, and the color of the tempeh is white. The biggest weight is the Grobogan var of 24.14 g. The largest water absorption capacity of Grobogan var is 188%. Therefore, the biggest swelling power of Grobogan var is 150%. Based on the time of fermentation, the highest water content of tempeh was found in Grobogan var (54 hours), with 67.33%. The highest ash content of tempeh was in Anjasmoro var (30 hours), with 1.97%. The highest fat content of tempeh was in Galunggung var (30 hours), with 8.89%. The highest protein content of tempeh was in the Galunggung var. (54 hours), with 25.17%. The highest carbohydrate content was in the Seulawah var (42 hours), with 11.43%.

Keywords: Chemical characteristics, fermentation time, *Glycine max*, seeds, soybean, tempeh, varieties

INTRODUCTION

According to botanists, the soybean is a plant from Manchuria and parts of China, where there are wide varieties of wild soybeans. Then it spreads to tropical and subtropical areas. After breeding, superior varieties of soybeans are produced. Soybean harvest age varies depending on the variety but generally ranges between 75 and 105 days. Regarding food and nutrition, soybean is the cheapest protein in the world and the highest protein compared to other beans (Septiani et al. 2004; Koswara 2005).

Soybean is the main raw material for making tempeh. So far, tempeh producers have met the needs of tempeh raw materials on imported soybeans, even though their price is increasing daily. According to Sisworo (2008), of the domestic demand for soybeans of two million tons per year, as much as 1.4 million tons are met from imports. If the world soybean price jumps above 100% from the normal Rp 2,500.00 per kg (August-September 2007), the soybean price will become Rp 7,500.00 per kg (Early January 2008).

Indonesia develops soybean varieties to overcome dependence on imported soybeans. The soybean varieties are expected to be processed into tempeh with better physical, chemical, and antioxidant activity than imported soybeans. The local soybean varieties used in this study were Grobogan, Argomulyo, Seulawah, Anjasmoro, Burangrang, and Galunggung. Indonesia has wide local soybean varieties due to individual variability. This individual variability is caused by internal factors, namely genetic factors, and external factors, namely the environment, such as soil conditions and types, nutrients, climate, temperature, humidity, and so on. Tempeh is one of the authentic Indonesian foods made from soybeans by fermentation. In the tempeh production process, the ingredients are boiled soybean seeds, microorganisms in the form of tempeh molds: *Rhizopus oligosporus, Rhizopus oryzae, Rhizopus stolonifer* (a combination of two/three species), and a supportive environment consisting of a temperature of 30°C, initial pH of 6, 8 and 70-80% relative humidity (Sarwon 1996).

According to SNI 01-3144-1992, soybean tempeh is a food product that is fermented from soybeans by certain molds, is in a compact solid form, and has a distinctive odor of white or grayish color (SNI 2010).

Tempeh is a highly nutritious food, so it has a strategic meaning and is very important for nutritional fulfillment. In addition, tempeh has other advantages: antioxidant content, simple producing technology, low price, good taste, and easy to cook.

The antioxidant content of tempeh can counteract free radicals, including vitamin E, carotenoids, superoxide dismutase, isoflavones, and so on. Therefore, the consumption of antioxidants in tempeh can mobilize antioxidant activity in the body. For example, there are three antioxidant compounds in soybeans in the form of isoflavone compounds: daidzein, genistein, and glycitein. They can prevent diseases caused by free radicals in the body, such as atherosclerosis, coronary heart disease, diabetes mellitus, cancer, etc., by serving as an antidote to free radicals (radical scavenger) (Haslina and Pratiwi 1996).

The use of several local soybean varieties as raw materials for making tempeh as an alternative to imported soybeans with the same physical and chemical characteristics with, or even better than, imported soybeans at an affordable price.

The aims of this study were: (i) to determine the effect of different varieties on the physical characteristics of the seeds of several soybeans (Grobogan, Argomulyo, Seulawah, Anjasmoro, Burangrang, and Galunggung), including weight, seed coat color, cooking quality such as swelling power, and water absorption; (ii) to determine the effect of fermentation time (30 hours, 42 hours, and 54 hours) on the chemical characteristics of tempeh (contents of protein, fat, water, ash and carbohydrates) from several soybeans (Grobogan, Argomulyo, Seulawah, Anjasmoro, Burangrang, and Galunggung).

MATERIALS AND METHODS

Place and time of research

This research was conducted in Pedan, Klaten and in CV. Chem-Mix Pratama, Kretek, Jambidan, Banguntapan, Bantul, Yogyakarta, Indonesia, within a period of 6 months.

Materials

The main ingredients in the making of tempeh are local soybeans, namely Grobogan, Seulawah, Burangrang, and Galunggung, and the introduced varieties Anjasmoro and Argomulyo obtained from the Balai Penelitian Kacang-Kacangan dan Umbi-Umbian (Malang, East Java, Indonesia), and Raprima tempeh yeast produced by PT. Aneka Fermentasi Industri (Bandung, West Java, Indonesia).

Research design

The experimental design of this study was a completely randomized design (CRD) with a factorial pattern consisting of 2 factors repeated 2 times. Factor 1: Soybean varieties (Grobogan, Argomulyo, Seulawah, Anjasmoro, Burangrang, and Galunggung) (K1, K2, K3, K4, K5, K6), so there were 36 replications. Factor 2: Duration of fermentation (30 hours, 42 hours, and 54 hours) (P1, P2, P3), so there were 36 replications.

Physical characteristics test of soybeans

Weight analysis was carried out by weighing 100 seeds using an analytical balance in duplicate. The color of the seed coat was observed visually. The observed cooking quality included (i) swelling power (Plhak et al. 1989) and (ii) water absorption (Plhak et al. 1989). Boiling quality analysis was performed by calculating each sample's weight (a gram) and volume (b mL). Furthermore, the seeds were put in a glass filled with water 10 times the volume of seeds and soaked for 12 hours. Then, the seeds were boiled with constant heat for 20 minutes. Finally, the seeds were drained, and the weight (d gram) and volume (e mL) were calculated.

Swelling power = e-b/a *x*100%
Water absorption = d-a/a *x*100%

Tempeh making

The stages of making tempeh were according to Syarief's (1999) method. First, seed sorting is done traditionally by choosing good and plump soybeans. In the container, soybean seeds are mixed with dirt, such as sand or wrinkled and porous seeds. The washing used clean running water. The immersion I used clean water as much as 500 mL for 12 hours. For the boiling I used 500 mL of clean water for 20 minutes. The immersion II used 500 mL of clean water for 12 hours. Stripping the epidermis was done by squeezing the soybean seeds. In making tempeh, soybean skin was added. Boiling II used 500 mL of clean water for 20 minutes. Draining was done by placing soybeans in a winnowing tray. Soybeans were inoculated using Raprima tempeh yeast. The packaging used banana leaves.

Fermentation was carried out with three kinds of treatment: 30 hours, 42 hours, and 54 hours.

Analysis method

According to Apriyantono et al. (1989), the analysis includes: (i) Moisture content using the gravimetric method. (ii) Ash content using a kiln. (iii) protein content using the micro-Kjeldahl method. (iv) Fat content using the Soxhlet method. (v) Carbohydrate content using the by difference method.

Data analysis

The research data were analyzed by SPSS, using analysis of variance (ANOVA) to determine whether there was a difference in treatment at the level of $\alpha = 0.05$. Then, DMRT (Duncan Multiple Range Test) was followed at the $\alpha = 0.05$ level.

RESULTS AND DISCUSSION

Physical characteristics

Table 1 shows that the colors of soybean seeds are yellow and greenish-yellow. Soybean seed coat color does not affect tempeh color. *Rhizopus* sp. needs energy and nutrients to change the yellow soybean seeds to become covered with white fungal mycelia to grow and develop.

The tempeh was white because fungal mycelia covered it. Good tempeh is characterized by a surface covered by mold mycelium (fine threads) evenly, compact and white. The soybean granules are filled with mycelium with strong and even bonds so that when the tempeh is sliced, the tempeh is not crushed.

The seed weight of 100 soybeans ranges from 7.66 g-24.14 g and varies for several soybean varieties. In general, the weight of 100 seeds was 7-10 g and was not significantly different for Argomulyo, Anjasmoro, and Burangrang varieties. The biggest weight was the Grobogan variety at 24.14 g, and the smallest was the Seulawah variety at 7.66 g. The seed size influences the amount of produced tempeh; therefore, a large seed size will produce more tempeh.

The highest water absorption was in Grobogan soybean with 188%, and the lowest was in Seulawah soybean with 106% (Table 1). All varieties showed significant differences. During soaking, soybeans will absorb water. The immersion temperature affects the rate of water absorption by soybean seeds. The higher the immersion temperature, the greater the rate of water absorption. On the other hand, the water absorption rate will decrease in proportion to the increase in the moisture content of soybean seeds. It seems that the absorption rate is also not significantly affected by the state of the soybean seeds.

Water absorption by legume seeds is very important concerning its utilization business. The amount of water absorption has something to do with softening of legumes. According to Kamil in Handajani and Atmaka (1993), several factors that affect the rate of water absorption are seed coat/seed membrane permeability, solution concentration, temperature, hydrostatic pressure, the surface area of seeds in contact with water, intermolecular forces, species, variety, maturity level, chemical composition, and the age of the seeds. Meanwhile, according to Bewley and Black in Handajani and Atmaka (1993), several factors that affect water absorption are seed coat anatomy, the external environment (soil, light, moisture), genetic factors, and other factors, including seed size.

It can also be seen in Table 1 that the highest swelling power value was 150% of Grobogan soybeans or 1.5 times the initial volume of soybeans. Table 1 also shows that the lowest swelling power value was 82% for the Seulawah variety soybean. All varieties showed significant differences. The swelling power of legumes increases the seed volume because the absorbed air is replaced by water during water absorption. In this case, the swelling power is determined more by the swelling of the seed coat and not by the seed's flesh, so the seed's softening will occur.

The correlation between water absorption and swelling power shows that the greater the water absorption capacity, the greater the swelling power. Therefore, according to Nabessa et al. in Handajani and Atmaka (1993), seeds swell during water absorption, increasing seed volume.

Water content

Water content is one of the most important characteristics of foodstuffs because water can affect the appearance, texture, and taste of foodstuffs. The water content in foodstuffs determines the freshness and durability of these foodstuffs. High water content makes it easy for bacteria, molds, and yeasts to breed, so there will be changes in foodstuffs (Wiryadi 2007). Based on the results of the study, the water content (%) in several soybean varieties with several fermentation time treatments is shown in Table 2. The water content of tempeh ranges from 55.80-67.33% and varies with the treatment of fermentation time and differences in soybean varieties. Table 2 shows that the longer the fermentation time, the higher the water content of tempeh for several soybean varieties.

All fermentation times showed significant differences, except for the Galunggung variety at 42 and 54 hours. In the Seulawah variety, 42 hours of fermentation decreased. The decrease was caused by the water content, which was still quite large at the time of immersion, making it difficult to drain these small and hard seeds.

The highest water content was in Grobogan soybean tempeh, with a fermentation time of 54 hours, which was 67.33%. The lowest water content was in Burangrang soybean tempeh, with a fermentation time of 30 hours, 59.03% (Table 2). The water content of tempeh increased with increasing fermentation time. According to Sudarmadji (1977), soybeans experienced an increase in water content after 40 hours of fermentation during the tempeh process.

Table 1. Physical characteristics of seeds of several soybean varieties

Varieties	Characteristics			
	Seed coat color	Weight of 100 seeds (g)	Water absorption (%)	Swelling power (%)
Grobogan	Yellow	24.14^{d}	188^{f}	150^{f}
Argomulyo	Yellow	13.44^{b}	144^{c}	110^{c}
Seulawah	Greenish-yellow	7.66^{a}	106^{b}	82^{b}
Anjasmoro	Yellow	14.02^{b}	175^{e}	132^{e}
Burangrang	Yellow	13.44^{b}	157.5^{d}	120^{d}
Galunggung	Yellow	16.01^{c}	119^{a}	100^{a}

Note: Numbers in the same column followed by the same letter show no significant difference ($\alpha < 0.05$)

Table 2. Water content (%) of tempeh with various fermentation time

Varieties	Water content (%)		
	Fermentation time (hour)		
	30	42	54
Grobogan	64.73^{fg}	65.65^{i}	67.33^{j}
Argomulyo	65.77^{h}	65.96^{i}	66.24^{i}
Seulawah	64.77^{fg}	62.72^{d}	64.19^{ef}
Anjasmoro	64.58^{fg}	64.92^{h}	65.94^{i}
Burangrang	59.03^{a}	62.07^{c}	63.72^{e}
Galunggung	61.16^{b}	62.12^{c}	62.15^{c}

Note: Numbers followed by the same letter show no significant difference ($\alpha < 0.05$)

According to Steinkraus (1995), during the fermentation of tempeh, the water produced results from the breakdown of carbohydrates by microbes. According to Rokhmah (2008), water is a product of aerobic fermentation. During tempeh fermentation, microbes digest the substrate and produce water, carbon dioxide, and large amounts of energy (ATP). During fermentation, the Rhizopus mold will destroy the matrix between the bacterial cells so that the soybeans will become soft on the third day. In the next stage, the cells in soybeans are destroyed by water, which results from the breakdown of carbohydrates and causes tempeh to become mushy and watery (Syarief 1999).

Fermentation time is one of the most important factors causing the increase in water content, so with increasing fermentation time, the water content will also increase (Mulato and Widyotomo 2003; Wiryadi 2007).

Water, as one of the products of metabolism, is very influential on other components, including mold growth as microorganisms that play a role in tempeh fermentation (Rokhmah 2008).

Protein content

In this study, the protein content determination test was carried out using the Micro-Kjeldahl method, calculated as total N. The total protein content of tempeh from several soybean varieties with variations in the time of fermentation treatment can be seen in Table 3. The protein content of tempeh ranges from 16.65-25.19%, varies between treatments of fermentation time and differences in soybean varieties. In all varieties and fermentation time, it tended to increase but showed no significant difference, except for Argomulyo and Galunggung varieties, 42 and 54 hours of fermentation showed significant differences.

Galunggung soybean tempeh, with 54 hours of fermentation time, had the highest protein content of 67.33%, but tempeh was in an over-fermented condition, so it was not preferred. At the same time, the lowest protein content in soybean tempeh Grobogan during 30-hour fermentation was 16.65% (Table 3). Tempeh protein content increases as fermentation time increases (30, 42, and 54 hours). These results are per the opinion of Astuti et al. (2000). Due to processing soybeans into tempeh, the total nitrogen, cellulose, and ash content increase significantly.

Vitamin B complex formation occurs in soybean tempeh fermentation, except for thiamin, which decreases (Astuti 2000). Vitamin B_{12} is produced by the bacterium *Klebsiella pneumoniae*, a desirable microorganism that may be required in the natural tempeh fermentation process (Steinkraus in Steinkraus 1983). It is suspected that during fermentation, tempeh also undergoes the formation of vitamin $B_{12,}$ so the increase in the amount of protein is thought to come from the nitrogen in the vitamin B complex.

Many fungi are active during tempeh fermentation, but researchers generally assume that *Rhizopus* sp. is the most dominant fungus. The fungus that grows on soybeans produces enzymes that can break down complex organic compounds into simpler compounds so that these compounds can be quickly used by the body (Pangastuti and Triwibowo 1996). In addition, *R. oligosporus* produces protease enzymes. The breakdown of protein complex compounds into simpler compounds is important in tempeh fermentation. Furthermore, it is one of the main factors determining the quality of tempeh, namely as a source of vegetable protein with a high digestibility value (Pangastuti and Triwibowo 1996).

Ash content

These mineral elements are also inorganic substances or ash content (Winarno 2002). According to Winarno (2002), ash is an inorganic substance from the combustion of organic material. Tempeh ash content ranged from 0.92-1.97% and varied during fermentation. The longer the fermentation time, the ash content of tempeh increases. Even though the ash content of the tempeh samples of the Grobogan variety decreased at 42 hours of fermentation, the decrease was not significant (Table 4).

There was no significant difference in all fermentation times except for the Argomulyo variety at 42 and 54 hours. Likewise, the Anjasmoro variety at 30 and 42 hours of fermentation showed a significant difference. The increase in ash content during tempeh fermentation is per Astuti et al. (2000), which stated that the total nitrogen content increased from processing soybeans into tempeh slightly, and the cellulose content and ash content increased significantly. This increase in ash content is probably due to the fermentation of molds producing enzymes for their metabolism. Enzymes are protein compounds containing the mineral element nitrogen (N), and the N is counted as ash.

Table 3. Protein content (%) of tempeh with various fermentation time

Varieties	Protein content (%)		
	Fermentation time (hour)		
	30	42	54
Grobogan	16.65^{a}	17.93^{abcd}	18.61^{cde}
Argomulyo	16.8^{ab}	18.28^{bcd}	21.06^{ghi}
Seulawah	17.70^{abc}	17.75^{abc}	18.69^{cde}
Anjasmoro	19.35^{def}	19.87^{efg}	20.21^{fgh}
Burangrang	20.21^{fgh}	21.51^{hi}	22.47^{ij}
Galunggung	21.96^{i}	23.47^{j}	25.19^{k}

Note: Numbers followed by the same letter indicate no significant difference ($\alpha < 0.05$)

Table 4. Ash content (%) of tempeh with various fermentation time

Varieties	Ash Content (%)		
	Fermentation time (hour)		
	30	42	54
Grobogan	1.29^{bc}	1.28^{bc}	1.34^{bcd}
Argomulyo	1.45^{cd}	1.69^{de}	1.97^{f}
Seulawah	0.99^{ab}	1.06^{ab}	1.24^{abc}
Anjasmoro	0.92^{a}	1.35^{bcd}	1.52^{cde}
Burangrang	1.34^{bcd}	1.47^{cd}	1.59^{cde}
Galunggung	1.29^{bc}	1.54^{cde}	1.84^{ef}

Note: Numbers followed by the same letter indicate no significant difference ($\alpha < 0.05$)

In addition, the increase in ash content is thought to come from vitamins formed by bacteria that grow during tempeh fermentation, such as *K. pneumoniae* (Ferlina 2009), especially vitamin B. Astuti et al. (2000), stated that during tempeh fermentation, the amount of vitamin B complex increased except for thiamin. As mentioned earlier, vitamin B12 is produced by the bacterium *K. pneumoniae* in the tempeh fermentation process (Steinkraus in Steinkraus 1983). During soybean fermentation, the increase in vitamin B_{12} levels can reach 33 times, the increase in riboflavin reaches 8-47 times, the increase in pyridoxine ranges from 4-14 times, the increase in niacin ranges from 2-5 times, the increase in biotin ranges from 2-3 times, the increase in folic acid ranges from 4-5 times and the increase in pantothenic acid reaches 2 times (Ferlina 2009). All these compounds contain the element nitrogen (N). Vitamin B_{12} also contains an atom of cobalt (Co) bonded similar to that of iron-bound in hemoglobin or magnesium in chlorophyll (Winarno 2002). Thus, the increase in ash is thought to come from nitrogen – nitrogen and cobalt (Co in vitamin B_{12}) contained in the vitamin B complex.

The lowest ash content was in Anjasmoro soybean tempeh at 30 hours of treatment with 0.92%, and the highest ash content was in Argomulyo soybean tempeh at 54 hours of treatment with 1.97%.

Fat content

Table 5 shows the total fat content of tempeh samples of several local soybean varieties such as Grobogan, Seulawah, Burangrang, Galunggung, and introduced varieties Anjasmoro and Argomulyo with variations in the length of fermentation treatment in this study ranging from 6.33-8.89%.

Table 5 shows that the fermentation treatment length affected the tempeh samples' fat content. The Grobogan, Argomulyo, and Galunggung varieties showed significant differences in all fermentation times. Meanwhile, the Anjasmoro variety showed a significant difference between 30 and 42 hours of fermentation. Meanwhile, the Burangrang variety showed a significant difference between 42 and 54 hours of fermentation. In other varieties and fermentation time, there was no significant difference.

In the Grobogan variety, fat content decreased at all fermentation times of 30, 42, and 54 hours, and all three showed significantly different. In the Argomulyo variety, fat content was decreased at all fermentation times of 30, 42, and 54 hours. At 30 hours and 42 hours of fermentation, there was no significant difference in fat content, while at 54 hours of fermentation, there was a significant difference.

The decrease in fat content also occurred in the Seulawah variety at all fermentation times of 30, 42, and 54 hours but did not show a significant difference. In the Anjasmoro variety, there was also a decrease in fat content at 30, 42, and 54 hours of fermentation. However, there was no significant difference between 42 hours and 54 hours of fermentation, while there was a significant difference between 30 hours of fermentation.

The decrease in fat content also occurred in the Burangrang variety at 42 and 54 hours of fermentation (8.43% and 7.87%), and there was a significant difference. In the Galunggung variety, there was a decrease in fat content at 30, 42, and 54 hours of fermentation, but there was no significant difference.

The fat content of tempeh in several local soybean varieties of Grobogan, Seulawah, Burangrang, and Galunggung and introduced varieties of Anjasmoro and Argomulyo with variations in the length of fermentation treatment (30, 42, and 54 hours) in this study tended to experience an insignificant decrease (Table 5). It is because fat is not easily used directly by microbes compared to protein and carbohydrates (Ketaren 1986; Wiryadi 2007). A significant decrease in fat content occurred in Anjasmoro tempeh at 42 hours of fermentation. In Kasmidjo (1990), it is stated that the fat content of soybeans will decrease due to fermentation into tempeh. More than 1/3 neutral fat from soybean was hydrolyzed by lipase enzyme during 3 days of fermentation by R.oligosporus at 37°C. After 48 hours of fermentation, all fat will be hydrolyzed.

In this study, it was found that the highest fat content was found in samples of tempeh of the Galunggung variety with a fermentation time of 30 hours (8.89%), while the lowest fat content was found in samples of tempeh of the Anjasmoro variety with a fermentation time of 54 hours (6.33%).

Carbohydrate content

Carbohydrate levels in tempeh from several soybean varieties with variations in the length of fermentation treatment can be seen in Table 6.

Table 5. Fat content (%) of tempeh with various fermentation time

Varieties	Fat content (%)		
	Fermentation time (hour)		
	30	42	54
Grobogan	8.22^{hi}	7.82^{f}	7.48^{e}
Argomulyo	8.31^{i}	8.19^{ghi}	7.39^{de}
Seulawah	7.24^{cde}	7.04^{bc}	6.87^{b}
Anjasmoro	8.40^{i}	6.52^{a}	6.33^{a}
Burangrang	8.43^{i}	8.43^{i}	7.87^{fg}
Galunggung	8.89^{j}	7.95^{fgh}	7.07^{bcd}

Note: Numbers followed by the same letter indicate no significant difference ($\alpha < 0.05$)

Table 6. Carbohydrate content (%) of tempeh with various fermentation time

Varieties	Carbohydrate content (%)		
	Fermentation time (hour)		
	30	42	54
Grobogan	9.11^{e}	7.32^{o}	5.24^{e}
Argomulyo	7.63^{m}	5.89^{f}	3.34^{a}
Seulawah	9.29^{p}	11.43^{r}	9.02^{n}
Anjasmoro	6.77^{j}	7.39^{l}	6.00^{g}
Burangrang	10.99^{q}	6.52^{h}	4.36^{c}
Galunggung	6.70^{i}	4.92^{d}	3.76^{b}

Note: Numbers followed by the same letter indicate no significant difference ($\alpha < 0.05$)

In the results of statistical analysis, it can be seen that the treatment of fermentation time and differences in soybean varieties have a significantly different effect on the carbohydrate content of tempeh samples. Furthermore, it can be seen from the different notations behind the carbohydrate content numbers.

The highest carbohydrate content was in the tempeh of Seulawah variety in 42 hours of treatment with 11.43%, and the lowest carbohydrate content was in the tempeh of Grobogan variety in 54 hours of treatment with 3.34%. According to Kim in Kasmidjo (1990), during the soaking process, monosaccharides increased, but in soaking for 24 hours at 25°C with a seed: water ratio of 1: 3 and 1:10, there was no decrease in oligosaccharides. According to Mulyowidarso (1988), sucrose decreased by 84%, while stachyose, raffinose, and melibiose decreased by 64% from the content in the seeds during soaking.

The reduction of stachyose, raffinose, and melibiose compounds and the increase of monosaccharides provide microbiological and nutritional advantages in the manufacture of tempeh. However, *R. oligosporus* cannot metabolize these compounds. On the contrary, it can utilize monosaccharides well. In addition, glucose is a sugar compound that encourages the germination of *R. oligosporus* spores.

Stachyose, raffinose, and sucrose, the main carbohydrate sources in beans, are carbon sources for tempeh yeast to grow. Therefore, the treatment of soaking and boiling can cause a reduction in the main sugar content. The decrease in carbohydrate levels during the fermentation process is thought to be due to the use of monosaccharides by tempeh yeast to grow so that the fermentation process can run. Stachyoses will be reduced further during fermentation by tempeh mushrooms, remaining only 30% of the stachyose content of raw soybeans after 48 hours and only 7% remaining after 72 hours of fermentation. Meanwhile, the relative raffinose content will be the same during fermentation.

Based on the research, it can be concluded that: (i) The difference in varieties does not affect the color of the tempeh produced. However, different varieties affect seed weight, water absorption, and swelling power of soybeans. Heavy seed weight will produce more tempeh. High water absorption will increase the swelling power. The soybean variety that has the best physical characteristics is Grobogan. (ii) Variations in the fermentation time treatment affect the chemical properties of tempeh. The longer fermentation time will cause the tempeh sample's water content, ash content, and total protein content to increase while the fat and carbohydrate content to decrease. The soybean variety that has the best chemical characteristics is Galunggung.

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Physiological responses of *Centella asiatica* to the herbicides of glyphosate and 2,4-D

GALIH SEPTIA AMIATI, SOLICHATUN♥, WIDYA MUDYANTINI
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: solichatun@staff.uns.ac.id

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Abstract. *Amiati GS, Solichatun, Anggarwulan E. 2019. Physiological responses of* Centella asiatica *to the herbicides of glyphosate and 2,4-D. Cell Biol Dev 3: 69-80.* The objective of this study was to determine the physiological response of *Centella asiatica* (L.) Urb. or *gotu kola* to the glyphosate herbicides and 2,4-Diclorophenoxyacetic acid (2,4-D). This study used a Completely Randomized Design (CRD) with two treatment factors: the type of herbicide and the dose of herbicide (0 $L.ha^{-1}$; 0.25 $L.ha^{-1}$; 0.5 $L.ha^{-1}$; 0.75 $L.ha^{-1}$; 1 $L.ha^{-1}$). The quantitative data obtained were examined by analysis of variance (ANOVA) to determine whether there was an effect of the treatments and followed by the DMRT (Duncan's Multiple Range Test) at a 5% level to determine the significant difference between the treatments. The t-test determined the effectiveness of glyphosate and 2,4-D. The results showed that the glyphosate herbicide at a dose of 0.25 to 1 L/ha did not inhibit the number of leaves, dry weight, and nitrate reductase. The glyphosate dose of 0.25 to 0.75 L/ha did not inhibit the formation of gross weight, chlorophyll content, and respiration rate. The glyphosate dose of 0.25 to 0.5 L/ha did not inhibit the leaf area and carotenoid content of *C. asiatica*. Herbicide of 2,4-D at a dose of 0.25 to 1 L/ha did not inhibit chlorophyll, respiration rate, and nitrate reductase. The glyphosate dose of 0.25 to 0.5 L/ha did not inhibit the formation of the number of leaves, leaf area, gross weight, dry weight, and carotenoid content. The glyphosate herbicide gave a lower inhibitory effect than the herbicide of 2,4-D in terms of growth: number of leaves, leaf area, gross weight, and dry weight. Cultivation of *C. asiatica* is more suitable for using the glyphosate herbicide because the growth of *C. asiatica* generally remains good. The glyphosate herbicide at a dose of 0.25 $L.ha^{-1}$ gave the highest yield on the formation of *C. asiatica* biomass.

Keywords: 2,4-Diclorophenoxyacetic acid, *Centella asiatica*, glyphosate, physiological

INTRODUCTION

Gotu kola (*Centella asiatica* (L.) Urb.) is a well-known medicinal plant worldwide. *C. asiatica* belongs to the Umbelliferae family, which is known as daun kaki kuda, pegagan, or antanan in Indonesia. *C. asiatica* is a cosmopolitan plant because it has a wide distribution area, particularly in the tropics and subtropics (Dalimartha 2000). *C. asiatica* is originally from tropical Asia and grows wild at an altitude of 1 to 2,500 m above sea level, in low shade fertile soil, foggy locations, along rivers, between rocks, meadows, yards, and on roadsides (Sudarsono et al. 2002).

Centella asiatica is often regarded as a neglected weed. However, many people have used *C. asiatica* as a medicinal plant. In West Java, *C. asiatica* leaves are also served as fresh vegetables, which cleanse the blood and cure digestive disorders (Steenis 1997); some even mix it as pickles (Dalimartha 2000). *C. asiatica* is also a cover crop to prevent erosion (Musyarofah 2006).

All parts of the *C. asiatica* can be used as traditional medicine. The *C. asiatica* herb cleanses the blood, improves blood circulation, is diuretic, antipyretic, hemostatic, improves memory nerves, anti-bacterial, anti-inflammatory, hypotensive, insecticidal, and inhibits excessive scar tissue (Sudarsono et al. 2002). In Brasilia, known as paardevoet, it is used to cure colon cancer. In Australia, known as *C. asiatica*, it is useful as an anti-senile and anti-stress. In China, known as ji xue cao, it is useful for improving blood circulation, and it is even believed to be more useful than ginko biloba or ginseng in Korea (Januwati and Yusron 2005).

The many benefits of *C. asiatica* seem to be related to the high content of active compounds, including asiaticoside, thankuniside, isothankunicide, madecasoside, brahminoside, brahmic acid, madekasitic acid, hydrocotyledon, mesoinoside, centellose, carotenoids, mineral salts (such as potassium, sodium, magnesium, calcium, iron salts), bitter substance vellarine and tannin (Dalimartha 2000). This plant is in great demand by traditional herbal medicine companies that process it into herbal ingredients. One herbal medicine factory requires approximately 100 tons of *C. asiatica* per year (Januwati and Yusron 2005); if only depending on the natural harvest, it is not enough to meet the demands of this plant. These conditions encourage the development of large-scale *C. asiatica* cultivation. A study on *C. asiatica* cultivation at the Center for Agricultural Research and Development resulted in total production of around 15-25 tons/ha or equivalent to 1.5-2.5 tons/ha of the dry plant. Further research in cultivation, such as the effect of harvesting system, the effect of shade level, type, and dose of fertilizer, has been carried out, while the growth of weeds and diseases has not been reported (Januwati and Yusron 2005).

Weed control in *C. asiatica* cultivation is currently only done manually, which has many weaknesses, such as

requiring more workforce, especially on large lands with high weed populations; it must be carried out more than once because manual control does not kill weeds, so production costs are increasing (Januwati and Yusron 2005). Chemical control also has negative effects, including types of herbicides that are not selective; in addition to killing weeds, it can also kill cultivated plants; the presence of herbicide residues left in the soil can cause plants to become poisoned even die (Anwar 2002).

Using herbicides properly and correctly can reduce the negative effects of chemical control. The selection of the glyphosate herbicides and 2,4-D to control weeds in *C. asiatica* cultivation was based on the advantages of these two herbicides. The advantages of the herbicide of glyphosate include: (i) it is non-selective with a broad spectrum, post-emergence systemic, which can control seasonal and annual weeds until their roots die after the plant grows (Moenandir 1988a,b), (ii) improving physical and chemical properties of soil because the use of the herbicide of isopropyl amine glyphosate can increase the rate of permeability, availability of P and CEC (Cation Exchange Capacity) of the soil, (iii) increasing the availability of organic N and C as well as soil microbes (Niswati et al. 1995), (iv) able to control narrow and broadleaf weeds that usually grows around *C. asiatica*. The advantages of herbicide of 2,4-D include: (i) it is selective and systemic in pre-emergence, (ii) it does not damage the environment because it is one of the auxin hormones belonging to phenoxy, (iii) able to control broadleaf weeds, and grass-like plants that usually grow around *C. asiatica* (Tjionger's 2002).

To optimize yields in *C. asiatica* cultivation, information on the plant's response to the herbicides of glyphosate and 2,4-D is required. Based on the information, the herbicide with the most appropriate type and dose can be selected to optimize the cultivation results in the cultivation practice. The objectives of this study were: (i) to determine the physiological response of gotu kola (*C. asiatica*) to the glyphosate herbicide. (ii) to determine the physiological response of *C. asiatica* to the herbicide of 2,4-D.

MATERIALS AND METHODS

Location and time of the study

The experimental study was carried out at the Green House of Biology Sub Lab, Central Laboratory of Mathematics and Natural Sciences of Universitas Sebelas Maret, Indonesia.

Materials

The main material was *C. asiatica* stolon obtained from the Gondang Village area, Tawangmangu Sub-district, Karanganyar District, Central Java, Indonesia. Other ingredients include the herbicide of glyphosate and the herbicide of 2,4-D.

Research design

This study used a Completely Randomized Design (CRD) which was arranged in a factorial manner with 2 treatment factors consisting of 5 levels with 5 replications as follows:

The first factor is the dose of the herbicide glyphosate consisting of 5 levels, including:

H_1K_1: 0 $L.ha^{-1}$ of glyphosate (without herbicide)

H_1K_2: 0.25 $L.ha^{-1}$ of glyphosate (equal to 0.875 mL/polybag)

H_1K_3: 0.5 $L.ha^{-1}$ of glyphosate (equal to 1.75 mL/polybag)

H_1K_4: 0.75 $L.ha^{-1}$ of glyphosate (equal to 2.625 mL/polybag)

H_1K_5: 1 $L.ha^{-1}$ of glyphosate (equal to 3.5 mL/polybag)

The second factor is the dose of the herbicide 2,4-D consisting of 5 levels, including:

H_2K_1: 0 $L.ha^{-1}$ of 2,4-D (without herbicide)

H_2K_2: 0.25 $L.ha^{-1}$ of 2,4-D (equal to 0.875 mL/polybag)

H_2K_3: 0.5 $L.ha^{-1}$ of 2,4-D (equal to 1.75 mL/polybag)

H_2K_4: 0.75 $L.ha^{-1}$ of 2,4-D (equal to 2.625 mL/polybag)

H_2K_5: 1 $L.ha^{-1}$ of 2,4-D (equal to 3.5 mL/polybag)

So, there are 10 treatments in total.

Procedure

Preparation of the growing media

Dried soil and compost were mixed with the ratio of soil: compost = 2:1 (i). 2 kg of the soil-compost mixture was taken and then put in a polybag Φ 30 cm (ii).

Preparation of seeds and treatments

Centella asiatica stolons, with 2 buds with a size of ± 2cm, were cut and then planted on the media provided (i). The herbicides were administered once for each treatment, which was done 7 before planting (Suwarni 2000) (ii).

Watering

Watering according to field capacity was carried out every day until new buds appeared and were 1 month old.

Harvest

Harvesting was done after *C. asiatica* had been grown for 2 months.

Observed variables

Leaf area

Observation of leaf area was carried out using a leaf area meter or gravimetric calculations. Leaf area was calculated by estimating by weight ratio (gravimetry). This can be done by, first, putting the leaf to be estimated on a piece of paper and producing a replica of the leaf. The leaf replica is cut out of paper whose weight and area are measured. The leaf area is then estimated based on the ratio of the weight of the leaf replica to the total paper:

$$LD=Wr/Wt \times LK$$

Where:

Wr: leaf replica paperweight

Wt: total papepaperweight

LK: total paper area (Sitompul dan Guritno 1995)

Number of leaves

The number of leaves is calculated at the end of the observation by calculating each sample plant's total number of leaves.

Gross weight

Observations are made by removing and cleaning the plants from the soil attached to the roots and then weighing them. Gross weight is weighed at the end of the observation using a balance.

Dry weight

Harvested crops cleaned of soil residues are put in paper bags and heated in the oven (temperature 50°C) for 4-5 days until a constant weight is reached. The constant weight achieved after being put in the oven is the dry weight of the plant.

Chlorophyll and carotenoid contents

Analyses of chlorophyll and carotenoid follow the method of Hendry and Grime (1993).

Nitrate reductase

Nitrate reductase analysis follows the Listyawati method (1994).

Respiration rate

Respiration rate analysis follows the Suwarsono method (1987).

Data analysis

Observational data are analyzed by analysis of variance (ANOVA). If there is a significant difference, it is followed by Duncan's multiple distance test (DMRT) at a 5% level. The t-test can determine the effectiveness of the herbicides of glyphosate and 2,4-D.

RESULTS AND DISCUSSION

Based on the analysis of variance of all observation variables, it was obtained that 5 of 8 observational variables of the treatment of herbicide administration of 2,4-D showed significant differences. In comparison, 7 of 8 observational variables of glyphosate showed significant differences. Treatment with the herbicide of 2,4-D had a significant effect on the number of leaves, leaf area, gross weight, dry weight, and carotenoid content. Treatment with glyphosate significantly affected the number of leaves, leaf area, gross weight, dry weight, chlorophyll content, carotenoid content, and respiration rate. The response rates of *C. asiatica* on the treatment of the herbicides of 2,4-D and glyphosate are presented in Table 1.

Number of leaves

Observation of the number of leaves was required in addition to being an indicator of growth as well as supporting data to explain the growth processes that occurred, such as the formation of plant biomass. The number of leaves increased with the age of the plant.

The analysis of variance showed that the dose of the herbicides of 2,4-D and glyphosate had a significant effect on the number of leaves of each plant. The average number of leaves per plant in the treatment of the herbicide of 2,4-D and glyphosate is presented in Tables 2 and 3.

Table 2 shows that the effect of the administration of the herbicide of 2,4-D up to a dose of 0.5 $L.ha^{-1}$ was not significantly different from that of the control plant (0 $L.ha^{-1}$). Increasing the dose of herbicide of 2,4-D caused a decrease in the number of *C. asiatica* leaves. In the treatment of the herbicide of glyphosate (Table 3), herbicide doses of 0.5 $L.ha^{-1}$ to 1 $L.ha^{-1}$ were not significantly different compared to the control plant. The number of leaves in glyphosate treatment at a dose of 0.25 $L.ha^{-1}$ provided the highest yield and was significantly different compared to the control plant.

Based on Figures 1 and 2, it can be seen that an increase in the dose of the herbicide of 2,4-D caused the number of *C. asiatica* leaves to be reduced. According to Moenandir (1990), the herbicide of 2,4-D can cause weakening of the root cortex. The weakening of the root cortex results in the inhibition of the absorption of nutrients, especially nitrogen, which is used as the main ingredient for leaf formation.

Table 1. Responses of analysis of variance of *C. asiatica* to the herbicides of 2,4-D and glyphosate

Herbicide	NL	LA	GW	DW	CL	CR	RR	NR
2,4-D	*	*	**	*	ns	*	ns	Ns
Glyphosate	*	**	*	*	*	**	**	Ns

Notes: NL: number of leaves, LA: leaf area, GW: gross weight, DW: dry weight, CL: chlorophyll, CR: carotenoids, RR: respiration rate, NR: nitrate reductase enzyme, ns: non-significant, *: significant, **: high significant

Table 2. The average number of *C. asiatica* leaves on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	39.33^{a}	30.33^{ab}	25.33^{abc}	21.67^{bc}	11.00^{c}

Table 3. The average number of *C. asiatica* leaves on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	31.00^{b}	42.67^{a}	31.00^{b}	23.67^{b}	23.00^{b}

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

Abidin (1994) stated that 2,4-D acid is one of the growth regulators classified as auxin. The role of auxin is to stimulate the division and enlargement of cells in plant shoots and cause the growth of new shoots. The addition of a higher amount of auxin, or the addition of a more stable auxin, such as 2,4-D acid, tends to cause callus growth of explants and inhibit plant shoot regeneration (Wetherell 1982). Suryowinoto (1996) states that 2,4-D acid can create mutations at a certain dose. According to Wattimena (1988), 2,4-D acid has high phytotoxicity properties, so that it can be an herbicide.

The glyphosate herbicide (Figures 1 and 2) generally resulted in a higher number of leaves formed than the herbicide of 2,4-D. This is because the addition of glyphosate is considered to be able to increase the availability of nitrogen in the soil. According to Niswati et al. (1995), tillage cultivation with the application of the glyphosate herbicide led to the availability of organic N and C and increased soil microbes. This means that glyphosate can contribute total N to the soil because glyphosate contains NH_2^+ groups. As stated by Rinsema (1983), leaf formation is determined by the availability of nitrogen, so it is possible to increase the number of *C. asiatica* leaves depending on the supply of nutrients in the soil.

The results of the study on the effect of the herbicides of glyphosate and legin on the nodulation behavior of *Arachis hypogaea* L. showed that glyphosate up to a dose of 4.5 L.ha^{-1} did not suppress plant growth and root nodule formation and showed the highest yield (Suwarni et al. 2000). A dose of 0.5 L.ha-1 to 1 L.ha-1 showed a decrease in the number of leaves. This is because administering glyphosate at high doses is not beneficial for root growth in absorbing nutrients in the soil. According to Suwarni et al. (2000), glyphosate inhibits root elongation because the entry of herbicides through the roots inhibits root growth and elongation and prevents lateral root growth. Active herbicides in the root system cause stunting and suppress lateral root growth (Moenandir 1993).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the area of *C. asiatica* leaves when administered the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* has the same response to variations in the types and doses of herbicides.

Leaf area

Leaves function as light receivers and photosynthetic tools. The rate of photosynthesis per unit plant can be determined by leaf area (Sitompul and Guritno 1995). The analysis of variance showed that the herbicides of 2,4-D and glyphosate had a significant effect on the leaf area. The average leaf area per plant in the treatment variations of the herbicides of 2,4-D and glyphosate is presented in Tables 4 and 5.

Table 4 shows that the herbicide treatment up to a dose of 0.5 L.ha^{-1} was not significantly different from those without the herbicide of 2,4-D. Leaf area at 0.75 L.ha^{-1} and 1 L.ha^{-1} herbicide treatments showed a significant decrease in leaf area compared to the control plant. On administering the glyphosate herbicide (Table 5), the herbicide dose of 0.25 L.ha^{-1} was followed by an increase in leaf area per plant. The leaf area in herbicide treatment at a dose of 0.25 L.ha^{-1} showed the highest significant yield compared to the control plant. At doses of 0.5 L.ha^{-1} to 1 L.ha^{-1} of glyphosate, the results were not significantly different compared to the plant without herbicide administration.

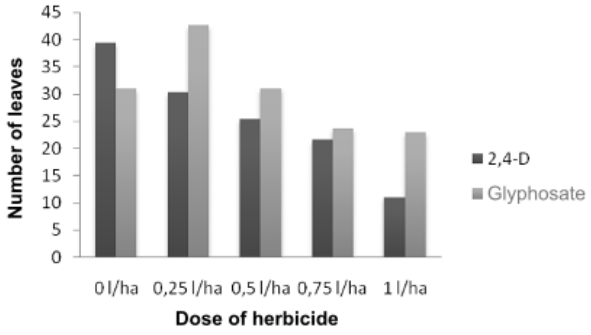

Figure 1. The effect of the herbicides of 2,4-D and glyphosate on the number of *C. asiatica* leaves

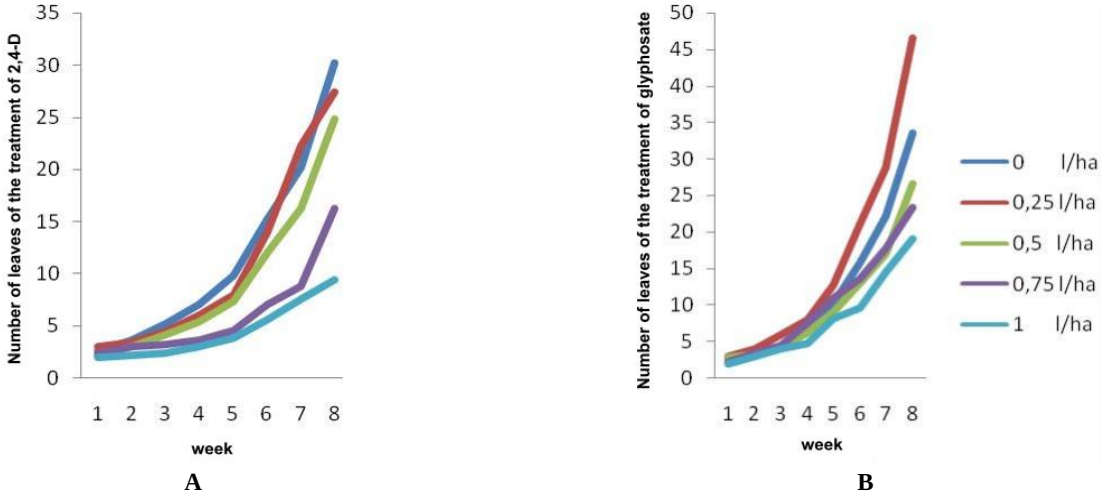

Figure 2. The effect of herbicides of 2,4-D (A) and glyphosate (B) on the number of *C. asiatica* leaves every week

Table 4. The average leaf area (cm^2) of *C. asiatica* on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	).5 L/ha	0.75 L/ha	1 L/ha
2,4-D	381.149[a]	300.207[a]	!13.972[ab]	96.884[b]	78.359[b]

Table 5. The average leaf area (cm^2) of *C. asiatica* on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	338.800[a]	381.890[a]	286.200[ab]	164.270[bc]	79.328[c]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

Auxin activity that is high enough causes abnormal growth in *C. asiatica*. It treated with 2,4-D was stunted with paler leaves. This is due to the location of the main activity of the herbicide of 2,4-D, which can change the growth pattern rapidly, so that the parenchyma cells of the roots swell, resulting in callus tissue and expansion of root primordia. Root elongation stops, and root tips swell.

Figure 3 shows that with an increase in the dose of the herbicide of 2,4-D, the area of the *C. asiatica* leaf formed decreased. The decrease in leaf area is caused by the lack of nutrients, especially nitrogen that plants can absorb, affecting plant growth, particularly leaf formation. Barriers in the process of nutrient absorption are caused by auxin activity, inhibiting root elongation, and swelling of root tips. According to Moenandir (1988a,b), the herbicide of 2,4-D treated on *Cyperus* sp (monocot) greatly reduced tissue differentiation and resulted in vacuolation with little cytoplasm. The size of the vascular system also decreases. In *Phaseolus* sp (dicot), leaf tissue is differentiated, and the replacement tissue has highly vacuolated cells without chloroplasts. 2,4-D also causes changes in morphology and internal structure of chloroplasts, damage to epidermal membrane cells, palisade, mesophyll, and changes in the metabolic system that greatly affect abscission events.

The herbicide of glyphosate (Figure 2) generally resulted in a wider leaf area than the herbicide of 2,4-D. This is because adding glyphosate can contribute total N into the soil derived from the NH_2^+ group of glyphosate. Added by Niswati et al. (1995), tillage cultivation with the application of the glyphosate herbicide leads to the availability of organic N and C and increased soil microbes. As Rinsema (1983) stated, leaf formation is determined by nitrogen availability. The increase in *C. asiatica* leaf area depends on the supply of nutrients in the soil. A dose of 0.5 $L.ha^{-1}$ to 1 $L.ha^{-1}$ showed decreased leaf area. This is because the absorption capacity of the soil has reached its maximum, so excess glyphosate accumulates in the area around the roots.

According to Moenandir (1993), glyphosate at high doses inhibits root elongation of sprouts because the entry of the herbicide of glyphosate into plants through roots inhibits root elongation and prevents lateral root growth. According to Thompson (1979), very little glyphosate herbicide is free in groundwater and immediately degraded by soil microorganisms. Adding a dose of glyphosate will inhibit the bacteria *Rhizobium* and *Pseudomonas* sp. in degrading glyphosate. This is because these microorganisms have EPSPS enzymes to produce aromatic amino acids in their bodies. Meanwhile, glyphosate inhibits the action of the EPSPS enzyme (5-enolpyrufil-shikamat-3-phosphate synthase) in plant tissues. Therefore, glyphosate also kills most soil microorganisms with the EPSPS enzyme, which forms aromatic amino acids, such as tryptophan, tyrosine, and phenylalanine (Wardoyo 2008).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the area of *C. asiatica* leaves when administrated the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* has the same response to variations in the types and doses of herbicides.

Gross weight

The number and area of leaves will affect the fresh weight of the plant. Fresh weight is also affected by water uptake by plants (Sitompul and Guritno 1995). According to Gardner et al. (1991), fresh weight is reflected by plants' water absorption in the soil. One of how plants absorb water depends on the soil's water.

Mass gain is often determined by harvesting the whole plant or desired part and weighing it quickly before too much water evaporates from the material. This fresh mass varies somewhat depending on the water status of the plant (Salisbury and Ross 1995). Fresh weight describes the water content and humidity of the plant at that time (Foth 1994). The average gross weight per plant in the treatment variations of the herbicides of 2,4-D and glyphosate is presented in Tables 6 and 7.

Analysis of variance (Table 6) showed that the application of the herbicides of 2,4-D and glyphosate significantly affected gross plant weight. In the treatment of the herbicide of 2,4-D at doses of 0.25 $L.ha^{-1}$ and 0.5 $L.ha^{-1}$, the dry weight was not significantly different from the control plant (0 $L.ha^{-1}$). Increasing herbicide doses of 0.75 $L.ha^{-1}$ and 1 $L.ha^{-1}$ showed a significant decrease in gross weight compared to the control plant. On administering the glyphosate herbicide (Table 7), the herbicide dose of 0.25 $L.ha^{-1}$ showed the highest yield of gross plant weight. The gross weight of the glyphosate herbicide treatment at doses of 0.75 $L.ha^{-1}$ and 1 $L.ha^{-1}$ showed a significant decrease in gross weight compared to no glyphosate herbicide.

Figure 4 shows that increasing the dose of 2,4-D caused the gross weight of *C. asiatica* produced to be lower. The herbicide of 2,4-D has an activity like auxin, which plants need for growth and development, in very small amounts. Administration of the herbicide of 2,4-D in high doses results in abnormal cell division and enlargement. According to Moenandir (1990), adding the herbicide of 2,4-D in seedlings will change the growth pattern quickly, where meristematic cells will stop dividing, and cell elongation stops long growth but continues radial expansion. In mature plants, the parenchyma cells swell and divide, resulting in callus tissue and expansion of root primordia. Root elongation stops, and root tips swell.

Table 6. The average gross weight (g) of *C. asiatica* on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	21.078[a]	19.167[a]	17.011[a]	5.047[b]	3.143[b]

Table 7. The average gross weight (g) of *C. asiatica* on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	16.414[ab]	22.062[a]	15.574[ab]	10.861[bc]	5.158[c]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

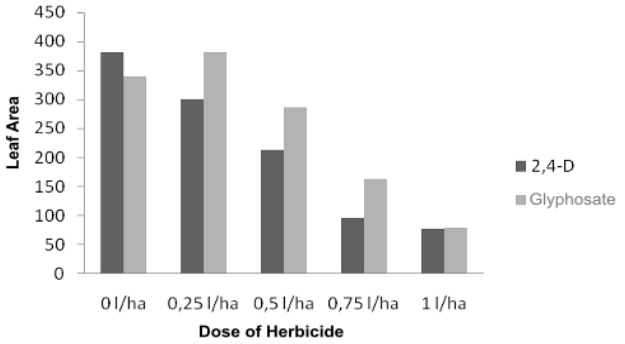

Figure 3. The effect of herbicides of 2,4-D and glyphosate on the surface area of *C. asiatica* leaves

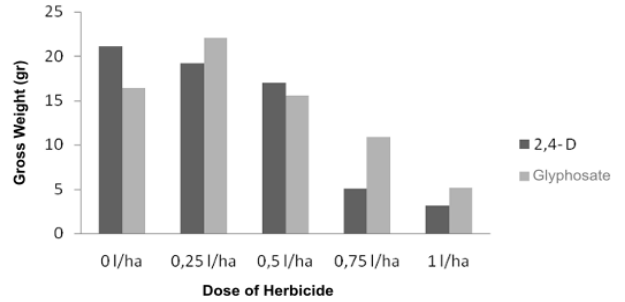

Figure 4. The effect of the herbicides of 2,4-D and glyphosate on the gross weight of *C. asiatica*

Swelling of the root tips will inhibit the absorption of water and nutrients from the soil resulting in disturbed plant metabolism. Thus, the fresh weight of the plant will also be reduced. The value of gross weight is influenced by tissue moisture content, nutrients, and metabolism (Salisbury and Ross 1995).

The glyphosate herbicide (Figure 3) generally resulted in a higher gross weight yield than the herbicide of 2,4-D. It is suspected that the dose of glyphosate increases nitrogen in the soil, so the absorption of nitrogen nutrients increases. The increased absorption of nitrogen nutrients will also increase the nitrogen content in the leaves. The nitrogen content of the leaf tissue will stimulate an increase in metabolic rate (Salisbury and Ross 1995). Added by Haryadi (1991), the enlargement of plant cells will form large cell vacuoles so that they can absorb large amounts of water. Besides, the formation of plant protoplasm will increase so that it can cause an increase in fresh weight and fresh yield of plants.

The increase in fresh weight is related to the number of leaves and leaf area. This is because increasing the number of leaves and leaf area on the plant will increase the fresh weight of the plant. According to Dwijoseputro (1993), fresh plant weight is influenced by nutrients in plant tissue cells. With the formation of roots and leaves, the physiological activities of plants in absorbing nutrients, water, and sunlight for the photosynthesis process can take place well in subsequent growth. The rapid growth of roots and leaves causes the absorption of nutrients, water, and light for more optimal photosynthesis; the resulting assimilation is used for faster plant development and the formation of more shoots so that the plant's fresh weight increases. At a dose of 0.5 L.ha^{-1} to 1 L.ha^{-1}, there was a decrease in fresh weight due to the addition of glyphosate at high doses, which could cause damage to the root system could inhibit the absorption of nutrients from the soil.

Based on the results of the t-test (Appendix IV), it can be seen that there was no significant difference in the gross weight of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Dry weight

To measure plant productivity, it is more relevant to use plant dry weight to measure growth (Salisbury and Ross, 1995). According to Lakitan (1993), the dry weight of plants reflects the accumulation of organic compounds that plants have successfully synthesized from inorganic compounds, especially water and carbon dioxide.

Dry weight is a balance between CO_2 uptake (photosynthesis) and CO_2 removal (respiration). If respiration is higher than photosynthesis, these plants lose dry weight and vice versa (Gardner et al. 1991). Added by Dwijoseputro's (1993) statement that 90% of plant dry matter results from photosynthesis and growth analysis expressed in dry weight. Table 8 shows the results of the average dry weight of *C. asiatica* plants.

Analysis of variance showed that the treatment with the herbicides of 2,4-D and glyphosate had a significant effect on the dry weight variable. Table 8 shows that the dry weight of plants in the treatment without the herbicide 2,4-D up to a dose of 0.5 L.ha^{-1} was not significantly different. The treatment with doses of 0.75 L.ha^{-1} and 1 L.ha^{-1} showed a significant reduction in dry weight compared to plants without the herbicide of 2,4-D. The dry weight of the herbicide treatment with glyphosate (Table 9) at a dose of 0.25 L.ha^{-1} showed the highest significant increase in dry weight compared to the control plant. Increasing the dose of herbicide from 0.5 L.ha^{-1} to 1 L.ha^{-1} showed a decrease in dry weight compared to plants without herbicides.

Table 8. The average dry weight (g) of *C. asiatica* on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	2.113[a]	1.691[a]	1.232[ab]	0.312[b]	0.215[b]

Table 9. The average dry weight (g) of *C. asiatica* on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	1.387[ab]	2.196[a]	1.190[ab]	0.994[b]	0.409[b]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

Figure 5 shows that the increase in the dose of the herbicide of 2,4-D caused the dry weight of the *C. asiatica* plant to decrease. According to Gardner et al. (1991), if respiration is higher than photosynthesis, the dry weight of the plant decreases, and vice versa. Figure 8 shows that the respiration rate of plants treated with high 2,4-D was the highest dose of the herbicide of 2,4-D. This is presumably due to a decrease in the rate of photosynthesis so that the dry weight produced is small at the increase in the highest herbicide dose. The increase in respiration rate aims to maintain the assimilate gradient (Moenandir 1990). The herbicide of 2,4-D causes damage to the phloem in the leaves. This results in the accumulation of assimilation in the leaves, so it is necessary to balance the assimilate gradient so that it is not excessive. Plants increase the rate of respiration with the aim that assimilation can be decomposed. Decomposition of assimilating in the form of starch aims to produce ATP or energy used for self-defense from excessive synthetic auxin activity. This causes the dry weight of *C. asiatica* to be produced a little at increasing the dose of the herbicide of 2,4-D.

The administration of the glyphosate herbicide (Figure 5) generally caused the dry weight of *C. asiatica* to remain higher than that of the herbicide of 2,4-D. This is presumably because the dose of glyphosate applied through the soil can increase nitrogen in the soil so that the absorption of nitrogen nutrients increases as well. The element N is always associated with an increase in the rate of photosynthesis. Chlorophyll and the enzyme ribulose bisphosphate carboxylase oxygenase (Rubisco) are the molecules that play a role in photosynthesis. Nitrogen is one element that plays a major role in synthesizing these two molecules. The increase in N levels in the soil due to glyphosate applied through the soil has a good effect on the photosynthesis process and will produce photosynthate, which is quite influential on the dry weight yield of plants. According to Al-Kaisi and Yin (2003), the overall response of N uptake by plants at various stages of growth has more effect on increasing plant dry weight than increasing N concentration in plant tissues.

The increase in dry weight at a dose of 0.25 L.ha^{-1} occurred due to the rate of photosynthesis in the form of photosynthate, which is the end product of the metabolic process. The end product of the photosynthesis process is sugar. Sugar is the basic material for preparing organic matter in plant cells, such as structural, metabolic, and important food reserves. Plant cell parts, such as cytoplasm, cell nucleus, and cell wall, are composed of organic matter. This process results in the accumulation of plant dry matter (Salisbury and Ross 1995). At doses of 0.5 L.ha^{-1} to 1 L.ha^{-1} of glyphosate, there was a decrease in dry weight. According to Suwarni et al. (1990), an increase in the dose of the herbicide of glyphosate causes peanut (*A. hypogaea*) plants to stunt their growth and root length. Therefore, the absorption of element N is not optimal and affects the process of photosynthesis and the resulting photosynthate. As a result, the dry weight of the plant decreases.

Based on the results of the t-test (Appendix IV), it can be seen that there was no significant difference in the dry weight of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Chlorophyll

Chlorophyll is a very important pigment in photosynthesis. It is a magnesium porphyrin attached to proteins. The relative amount of chlorophyll varies typically from one plant species to another (Lehningher 1990). Salisbury and Ross (1995) classified 2 pigments found in the thylakoid membrane, including green chlorophyll, which consists of chlorophyll a and chlorophyll b, and carotenoids which are yellow to orange pigments.

The analysis of variance in Tables 10 and 11 shows that the administration of the herbicide of 2,4-D did not have a significant effect. In contrast, the administration of the glyphosate herbicide significantly affected chlorophyll content. Based on Table 10, it can be seen that the increasing administration of the herbicide of 2,4-D showed an increase in plant chlorophyll content, and a dose of 1 L.ha^{-1} showed the highest average chlorophyll content but was not significantly different from the control plant. In administering the glyphosate herbicide (Table 11), herbicide doses of 0.25 L.ha^{-1} to 0.75 L.ha^{-1} showed no significant results from the control plant. Chlorophyll content at a dose of 1 L.ha^{-1} showed a significant decrease compared to without glyphosate herbicide.

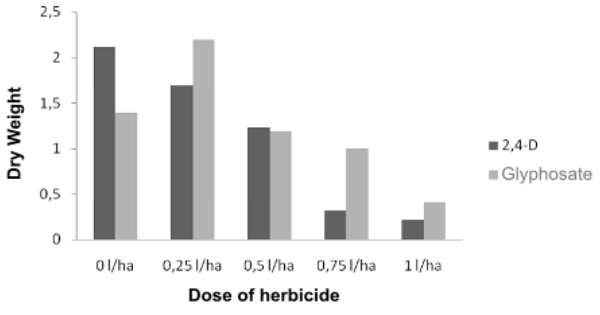

Figure 5. The effect of the herbicides of 2,4-D and glyphosate on the dry weight of *C. asiatica*

Table 10. The average chlorophyll content (mg/L) of *C. asiatica* on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	67.712	73.340	74.544	75.815	76.915

Table 11. The average chlorophyll content (mg/l) of *C. asiatica* on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	75.700[a]	75.452[a]	75.094[a]	73.004[a]	69.906[b]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

Based on the graph of the average chlorophyll content of *C. asiatica* (Figure 6), it is shown that the increase in the dose of the herbicide of 2,4-D caused the total chlorophyll content of the *C. asiatica* plant to be significantly high. This may be because the administration of the herbicide of 2,4-D does not significantly affect the metabolic process in the formation of chlorophyll. The increase in the amount of chlorophyll is considered to be influenced by the number of carotenoids. By the opinion of Salisbury and Ross (1995), in addition to functioning as light-taking pigments that are useful for photosynthesis, carotenoids also function to protect chlorophyll from damage caused by oxidation by oxygen. Another factor affecting the high levels of chlorophyll is the amount of nitrate reductase (Figure 9).

The higher the nitrate reductase, the higher the chlorophyll count. Nitrate reductase converts nitrate into ammonia which can then be converted into ammonium in the presence of protons. Ammonium combines with glutamate to be converted with glutamine synthase to be glutamine. Glutamine then binds to α-ketoglutarate with the help of glutamate synthase to convert to glutamate. Glutamate will produce proline, arginine, and δ-aminolevulinate. δ-aminolevulinate is an intermediate in forming chlorophyll (Loveless 1991; Salisbury and Ross 1995).

The administration of the glyphosate herbicide (Figure 6) generally resulted in lower chlorophyll content of *C. asiatica* than the herbicide of 2,4-D. This shows a similar pattern to the growth parameters. Chlorophyll plays an important role as a medium for capturing energy from sunlight which in photosynthesis will produce ATP and NADPH. According to Sampson et al. (2003) and Fracheboud (2006), chlorophyll content can be used as a sensitive indicator of the physiological condition of a plant because it is positively correlated with leaf nitrogen content. It can be used as an indicator of the rate of photosynthesis. The highest chlorophyll content was at a dose of 0.25 L.ha^{-1} because, at that dose, the N in the soil was available optimally due to the administration of glyphosate. Therefore, the amount of N that plants can absorb also increases. The high N absorbed by plants also increases chlorophyll content because chlorophyll molecules are composed of C, H, O, and N elements and one Mg atom (Gardner et al. 1991).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the chlorophyll content of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Carotenoids

Carotenoids are photosynthetic pigments divided into two groups: xanthophylls, such as lutein and zeaxanthin, and carotene, which consists of β-carotene and α-carotene (Zaripheh and Erdman 2002). Carotenoids have several functions for plants, including absorbing light and stabilizing the structure by disposing of excess energy. This component also protects plants from free radicals when the light intensity exceeds the capacity for photosynthesis (Frank and Codgell 1996; Havaux and Niyogi 1999). Carotenoid biosynthesis is influenced by pH, enzyme activity, light, oxidation, and water. The optimum temperature for carotenoid biosynthesis is around 30°C, while the required optimum pH is 7.4 (Salisbury and Ross 1995). The average chlorophyll content of *C. asiatica* in the treatment of the herbicides of 2,4-D and glyphosate is presented in Tables 12 and 13.

The analysis of variance (Tables 12 and 13) showed that the treatment with the herbicides of 2,4-D and glyphosate had a significant effect on carotenoid content. The treatment of the herbicide of 2,4-D (Table 12) at doses of 0.5 L.ha^{-1} to 1 L.ha^{-1} showed a significant increase in carotenoid content compared to the control plant. Chlorophyll contents at a dose of 0.25 L.ha^{-1} showed that there were not significantly different from the control plant. In the administration of the glyphosate herbicide (Table 13), herbicide doses of 0.5 L.ha^{-1} to 1 L.ha^{-1} showed a significant decrease in chlorophyll content compared with no administration of the herbicide of glyphosate.

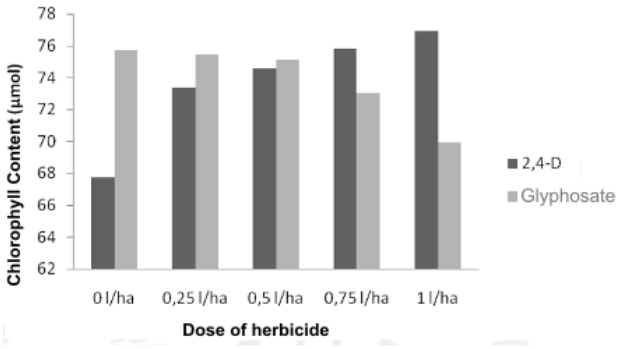

Figure 6. The effect of the herbicides of 2,4-D and glyphosate on the chlorophyll content of *C. asiatica*

Table 12. The average carotenoid content (µmol) of *C. asiatica* on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	0.517[b]	0.603[ab]	0.657[a]	0.664[a]	0.662[a]

Table 13. The average carotenoid content (µmol) of *C. asiatica* on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	0.667[a]	0.657[a]	0.614[b]	0.592[b]	0.588[b]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

Figure 7 shows that increasing the dose of 2,4-D caused the carotenoid content of the *C. asiatica* to be high. This may be due to the number of carotenoids in balance with the amount of chlorophyll. When the amount of chlorophyll is high (Figure 6), the number of carotenoids is also high and vice versa. High carotenoid content may be a form of self-defense. Salisbury and Ross (1995) opinion that carotenoids are isoprenoid polyene compounds that are lipophilic or insoluble in water, easily isomerized and oxidized, absorb light, reduce singlet oxygen, block free radical reactions, and can bind to hydrophobic surfaces. The increase in plant stress levels due to herbicide doses will also increase the number of plant carotenoids.

The glyphosate herbicide (Figure 7) generally resulted in lower carotenoid content formed compared to the herbicide of 2,4-D. This is due to damage to the roots causing a decrease in root activity in absorbing nutrients, so photosynthesis is disrupted. Both of these are caused by damage to the cell structure. The damage to the cell structure is preceded by damage to the cell membrane, followed by damage to cell organelles, such as chloroplasts, mitochondria, and the nucleus. The damage to each membrane also precedes these organelles, making its structure unclear (Einhelling 1995). Carotenoids are present in the plastid membrane and have a double membrane. One of the most important types of plastids is the chloroplast. If the chloroplast is damaged, the biosynthesis of carotenoids may be inhibited.

The biosynthesis of carotenoids begins with the formation of prenyl pyrophosphate in plant plastids, which are the precursors of carotenoid biosynthesis. Prenyl pyrophosphate is formed by prenyl transferase, which forms dimethylallyl pyrophosphate (IPP). Then, it was synthesized by geranyl-geranyl pyrophosphate (GGPP). The condensation of 2 GGPP molecules forms a pyrophosphate prephytoen as an intermediate (phytoene synthesis). Phytoene is formed by removing the pyrophosphate group. Furthermore, the conversion of phytoene into lycopene forms a variety of carotenoids (Hirschberg et al. 1997).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the carotenoid content of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Respiration rate

Plants carry out the process of photosynthesis and respiration. Respiration is a process of dismantling (catabolism or dissimilation) of stored chemical energy (carbohydrate organic substances resulting from photosynthesis) to carry out life processes such as the formation of organic substances, activities in absorption (osmosis), accumulation of salts, protoplasm drainage, cell division and other activities (Dwijoseputro 1993).

Analysis of variance showed that the application of the herbicide of 2,4-D did not significantly affect the respiration rate of each plant. Meanwhile, the administration of glyphosate affected the respiration rate significantly. The average respiration rates of the herbicides of 2,4-D and glyphosate are presented in Tables 14 and 15.

Table 14 shows that the herbicide treatment of 2,4-D showed the highest average respiration rate at a dose of 1 $L.ha^{-1}$. These results were not different from that of *C. asiatica* without herbicide. The administration of the herbicide doses was 0.25 $L.ha^{-1}$, 0.5 $L.ha^{-1,}$ and 0.75 $L.ha^{-1}$. The administration of the glyphosate herbicide (Table 15) at a dose of 1 $L.ha^{-1}$ showed the highest results and was significantly different from the control plant, doses of 0.25 $L.ha^{-1}$, 0.5 $L.ha^{-1,}$ and 75 $L.ha^{-1}$.

Table 14. The average respiration rate (ppm/l/min) on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	46.00	21.00	27.00	31.00	50.00

Figure 7. The effect of herbicides of 2,4-D and glyphosate on carotenoid content of *C. asiatica*

Table 15. The average respiration rate (ppm/l/min) on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	43.00[b]	21.00[c]	21.00[c]	26.00b[c]	75.00[a]

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

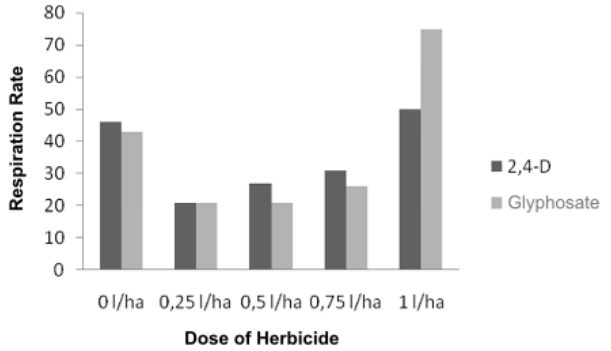

Figure 8. The effect of the herbicides of 2,4-D and glyphosate on the respiration rate of *C. asiatica*

The graph of the average rate of respiration of *C. asiatica* (Figure 8) shows that the use of the herbicide of 2,4-D shows that the respiration rate of *C. asiatica* is higher. This is because 2,4-D affects the mitochondrial membrane as a site for oxidative phosphorylation. Electrons penetrate the membrane so that there is no energy accumulation in the form of ATP (Moenandir 1990). The increase in respiration rate is considered to be due to the prevention of ATP synthesis by 2,4-D (non-combining group) so that it can stimulate respiration in phosphate-deficient media, supporting ATP hydrolysis. This follows the opinion of Nurjanah (2003) that herbicides with the active ingredient 2,4-D can inhibit weed growth by accelerating respiration. Increased respiration can result in starch being constantly overhauled to produce energy in self-defense. When starch is unavailable, energy is not produced for self-defense, and eventually, the plant dies. The increase in respiration rate aims to maintain the assimilate gradient in the form of glucose or starch (Moenandir 1990). The herbicide of 2,4-D causes damage to the phloem in the leaves. This results in the accumulation of assimilation in the leaves, so it is necessary to balance the assimilate gradient so that it is not excessive. Plants increase the rate of respiration with the aim that assimilation can be decomposed.

Administration of the dose of the glyphosate herbicide (Figure 8) in general caused the rate of respiration to be higher than that of the herbicide of 2,4-D. This is because plants carry out a defense system against glyphosate's active substance by forming ATP through glycolysis. The more doses of herbicide administrated, the more the defense system will work harder, and the respiration rate will be faster. Increased respiration is caused by low energy production; 2 ATP under anaerobic conditions from each glucose molecule compared to 36 ATP produced under aerobic conditions. Because cells still need NAD^+, the glycolysis process continues under anaerobic conditions due to the inhibition of O_2 uptake by glyphosate at high doses (Moenandir 1988a,b). The respiration rate must be significantly increased to meet the minimum needs (Delita et al. 2008).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the respiration rate of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Nitrate reductase

Nitrate reductase (NR) is one of the most sensitive plant enzymes studied. NR has been studied intensively because its activity often affects the rate of protein synthesis in plants that absorb NO_3^- as the main nitrogen source. NR activity is influenced by several factors, including the rate of synthesis and the rate of an overhaul by protein-destroying enzymes. Inhibitors and activators also influence it in the cell (Salisbury and Ross 1995).

The analysis of variance showed that the treatment with the herbicides of 2,4-D and glyphosate did not significantly affect the nitrate reductase content of plants. The average levels of nitrate reductase due to treatment with the herbicides of 2,4-D and glyphosate are presented in Tables 16 and 17.

Table 16 shows that for the treatment of the herbicide of 2,4-D, the highest average nitrite reductase content was at a dose of 1 $L.ha^{-1}$. These results were not significantly different from that of *C. asiatica* without herbicides, doses of 0.25 $L.ha^{-1}$, 0.5 $L.ha^{-1,}$ and 75 $L.ha^{-1}$. Meanwhile, in administering the glyphosate herbicide (Table 17), the highest average nitrate reductase content was at a dose of 0.75 $L.ha^{-1}$. These results were not significantly different from the control plant, with doses of 0.25 $L.ha^{-1}$, 0.5 $L.ha^{-1,}$ and 1 $L.ha^{-1}$. In administering the herbicide glyphosate, the highest average nitrate reductase content was at a dose of 0.5 $L.ha^{-1}$. These results were not significantly different from the herbicide administration at doses of 0 $L.ha^{-1}$, 0.25 $L.ha^{-1}$, 0.75 $L.ha^{-1}$, and 1 $L.ha^{-1}$.

Figure 9 shows that the application of the herbicide of 2,4-D caused high nitrate reductase content formed in the *C. asiatica*. According to Planchett (2004), nitrate reductase (ANR) activity is positively correlated to stress. As a result, plants carry out a biochemical response by increasing the efficiency of using nitrate as an alternative electron acceptor by reducing nitrate to nitrite by the enzyme nitrate reductase. This is due to swelling in the root area resulting in disturbed oxygen absorption through the roots and anaerobic conditions in the area around the roots. This anaerobic condition causes the reduction of nitrate to nitrite and is the only way to replace the role of oxygen in electron transport.

Table 16. The average nitrate reductase (µmol/g) on the administration of the herbicide of 2,4-D

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
2,4-D	28.182	17.870	28.959	74.306	59.46

Table 17. The average nitrate reductase (µmol/g) on the administration of the herbicide of glyphosate

Type of herbicide	Dose of herbicide				
	0 L/ha	0.25 L/ha	0.5 L/ha	0.75 L/ha	1 L/ha
Glyphosate	15.555	22.186	28.017	27.666	27.809

Note: Numbers followed by the same letter in the same row or column indicate no significant difference in the DMRT (Duncan's Multiple Range Test) at a 5% level

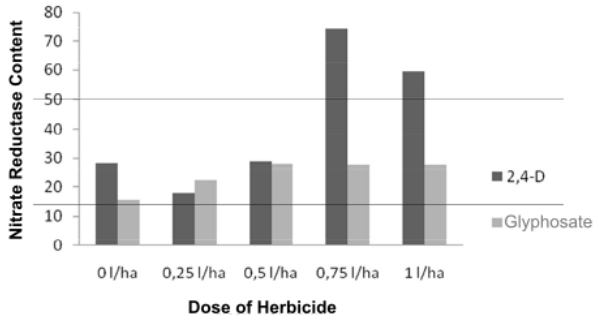

Figure 9. The effect of the herbicides of 2,4-D and glyphosate on nitrate reductase of *C.asiatica*

Glyphosate herbicide administration (Figure 9) generally resulted in lower nitrate reductase content formed compared to the herbicide of 2,4-D. Still, nitrate reductase content in glyphosate treatment of various concentrations was relatively stable compared to the control plant. This means that the glyphosate herbicide does not inhibit the formation of the enzyme nitrate reductase. According to Niswati et al. (1995), cultivation without tillage with the application of the glyphosate herbicide increases the availability of organic N and C and soil microbes. N availability in the soil will increase the nitrate reduction process that occurs in two different reactions. The first reaction is catalyzed by nitrate reductase, an enzyme that will transport two electrons from NADH or NADPH and produce nitrite. The second reaction of the whole nitrate reduction process is the conversion of nitrite to ammonium (NH_4^+) (Planchett 2004).

Nitrate reductase is an essential enzyme in the chain of nitrate reduction to ammonium which is useful in forming amino acids, proteins, and other compounds containing the element N (Levitt 1980). Various environmental and nutritional factors determine the amount of the enzyme nitrate reductase in an organism. Plants in an environment rich in nitrate will have a large amount of the enzyme nitrate. However, the amount of the enzyme will decrease if they are in an environment with a lot of ammonium ions (Linbald and Guerrero 1993).

Ammonium is the product of nitrate reductase that can catalyze the nitrate reduction process. If the amount of product continues to increase beyond the level of cell demand, the product will become a blocker. Many enzymes will be inactive until the product of the final compound is reduced in number. This mechanism is called the feedback mechanism as a fast and sensitive mechanism to avoid the excessive synthesis of a final product (Lakitan 1993).

Based on the results of the *t*-test (Appendix IV), it can be seen that there was no significant difference in the nitrate reductase content of *C. asiatica* in the administration of the herbicides of 2,4-D and glyphosate. This means that *C. asiatica* had the same response to variations in the types and doses of herbicides.

Based on the research that has been conducted, it can be seen that: (i) Administration of the glyphosate herbicide at a dose of 0.25 to 1 L/ha did not inhibit the number of leaves, dry weight, and nitrate reductase. The glyphosate dose of 0.25 to 0.75 L/ha did not inhibit the formation of gross weight, chlorophyll content, and respiration rate. The glyphosate dose of 0.25 to 0.5 L/ha did not inhibit the leaf area and carotenoid content of *C. asiatica*. (ii) The herbicide of 2,4-D up to a dose of 0.25 to 1 L/ha has not inhibited chlorophyll, respiration rate, and nitrate reductase. The glyphosate dose of 0.25 to 0.5 L/ha did not inhibit the formation of the number of leaves, leaf area, gross weight, dry weight, and carotenoid content. (iii) The effect of glyphosate herbicide showed a lower inhibitory effect than the herbicide of 2,4-D in terms of growth: number of leaves, leaf area, gross weight, and dry weight. Cultivation of *C. asiatica* is more suitable than using the herbicide glyphosate because the growth of *C. asiatica* is generally good.

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Growth inhibitory effect of *Conocarpus lancifolius* plant aqueous extract on *Fusarium oxysporum* causal agent of wilt in some crops

MAWADA AWAD SULIMAN ALI ELSHAIR, IBRAHIM SAEED MOHAMED♥
Department of Plant Protection, College of Agricultural Studies, Sudan University of Science and Technology. Shambat, Sudan. Tel.: +249-9-12422515, ♥email: ibrahsaeed@sustech.edu, ibrahsaeed@yahoo.com

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Abstract. *Elshair MASA, Mohamed IS. 2019. Growth inhibitory effect of* Conocarpus lancifolius *plant aqueous extract on* Fusarium oxysporum *causal agent of wilt in some crops. Cell Biol Dev 3: 81-85. Fusarium oxysporum* affects a wide variety of different ages tomatoes, tobacco, legumes, cucurbits, sweet potatoes, chickpea and Banana. The present investigation was undertaken to study the effect of Damas (*Conocarpus lancifolius* Engl.) plant parts (leaves, fruits, barks, and roots) aqueous extracts and fungicide Score (250 EC) on the growth of the fungus *F. oxysporum*, the causal agent of wilt disease in crops. Three concentrations of aqueous leaves, fruits, barks, and roots extract of *C. lancifolius*, each of 25, 50, and 100%, and fungicide was used in addition to control. The assessment of their inhibitory effect against the pathogen was recorded through the fungal growth. The results showed that all concentrations of the leaves, fruits, barks, and roots aqueous extracts *C. lancifolius* plant tested and fungicide showed significant inhibitory effect against the linear growth of *F. oxysporum* compared to control. Moreover, the concentration of each aqueous extract reacted differently against *F. oxysporum*. However, the highest concentration of the *C. lancifolius* extracts (100%) gave significantly higher inhibition zone percentages (75.5%, 68%, 66%, and 50%) than the untreated control. Among the *C. lancifolius* parts, extracts screened from the fruit (75.5) were more effective in suppressing the fungus growth than its equivalent other parts. The results showed that the antifungal activity increased with the extract concentration. The fungus *F. oxysporum* differs in its response to the different concentrations, but on the whole, growth inhibition increased with the concentration. The current results were considered promising and encouraging to carry out a phytochemical analysis of different parts of *C. lancifolius* plant using different solvents to determine the bioactive ingredient in each of these parts.

Keywords: *Conocarpus lancifolius*, damas plant, *Fusarium oxysporum*, growth inhibitory effect

INTRODUCTION

Fusarium oxysporum is a major cause of wilting (Nene et al. 1991). The disease is common in most tomato-producing countries and is a major disease. It is a disease transmitted by seeds and soil. The fungal pathogen *F. oxysporum* affects a wide variety of hosts of different ages tomatoes, tobacco, legumes, cucurbits, sweet potatoes, chickpeas, and bananas are among the most susceptible crops, but other herbaceous plants are also affected (Pan Germany 2010).

Fusarium wilt is a common causative agent of vascular wilt, which has similar symptoms to verticillium wilt. The causative agent of *Fusarium* wilt is *F. oxysporum* (Snyder and Hansen 1940). The species is further divided into forma specialists based on the host plant. These fungi typically produce symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping. The most important of these are vascular wilt. *F. oxysporum* is a common soil saprophyte and pathogen that feeds on dead and decaying organic matter. It survives in soil debris such as mycelium and all types of spores but is often recovered from the soil as chlamydospores (Snyder and Hansen 1940). It is a major wilt pathogen of many economically important crops. It is a soil-borne pathogen that can live in the soil for a long time, so rotational cultivation is not a useful control method. It can also spread through infected dead plant matter, so cleaning up late in the season is important. Members of *F. oxysporum* are found all over the world.

Before global transport, however, many of the different pathogen varieties had been isolated. Now world trade has spread *F. oxysporum* inoculums with the crop. A recent example of this is the spread of *F. oxysporum* f.sp. Cubense, may have originated in Asia and recently appeared in banana-growing areas in the South Pacific (Davis and Richard 2004). In Sudan, various diseases are known to limit agricultural production. One is *Fusarium* wilt caused by *F. oxysporum*, a major disease-causing economic loss (Bhatia et al. 2004). The disease is particularly severe in traditional production areas. Based on the above, this study was conducted to focus on research on two components for managing *Fusarium* wilt caused by *F. oxysporum*, superior plant extracts, and synthetic fungicides under laboratory conditions to formulate a promising disease management approach. With the following objectives: (i) To study the antifungal potential of some higher plants, crude extract against *F. oxysporum*. (ii) Evaluate the effect of the systemic fungicide on fungal growth. (iii) Development of promising components for *Fusarium* wilt disease management.

MATERIALS AND METHODS

This study was conducted under laboratory conditions at the Department of Plant Pathology, College of Agricultural Studies "Shambat," Sudan University of Science and Technology (SUST), Sudan, from November 2015 to February 2016 to evaluate the inhibitory effect of all parts of damas (*Conocarpus lancifolius* Engl.) (leaves, bark, fruit, and root) aqueous extracts and fungicidal efficacy, Score 250 EC, against the fungus *F. oxysporum.*

Collections of plant samples

Various parts of *C. lancifolius* (fruits, leaves, bark, and roots) were collected from trees growing in the Elshair Farm Project. Collected parts were cleaned of dust and foreign matter by hand, washed with distilled water, surface sterilized with 1% sodium chloride, washed thoroughly in sterilized water, and dried in the shade at room temperature, ground, and pulverized separately to obtain a fine powder for extraction and is preserved for use.

Preparations

Preparation of plant extract

All parts of *C. lancifolius* (leaves, bark, fruit, roots) were collected from the elshair farm project and dried in the shade. After the plants were completely dry, the plants were ground separately to obtain fine powder for extraction.

Preparation of inoculums

Pure cultures of *F. oxysporum* were prepared using 7-day-old mycelia. The fungi were grown on PDAs and then aseptically transferred to the center of Petri dishes containing PDA medium and incubated at 25°C. Linear growth of the fungus was determined in cm after 72 hours.

Aqueous extract preparation

Aqueous extracts of each plant material were prepared as recommended by (Okigbo and Ogbonnaya 2006). The fine powder obtained from various parts of *C. lancifolius* was weighed (100 g), and 100 mL of sterilized distillate was added to it in a 250 mL Erlenmeyer flask and then placed in a shaker for 24 hours. The extracts were filtered under reduced pressure as 100% raw water extract. The other concentrations obtained were diluted to 50% and then 25% and stored in the refrigerator to serve as stock solutions.

Preparation of fungicide

The fungicide tested was Score of which 2 mL were dissolved in 1000 mL of sterilized distilled water to give 100 ppm.

The effect of each extract was calculated as the percentage of reduction in diameter of fungal growth (R) where:

$$R = \frac{dc - dt}{dc} \times 100$$

Where R = Percentage reduction of the growth, dc = diameter of controlled growth, and dt = diameter of treated growth

Effect of different parts of *C. lancifolius* extract on the linear growth of the *F. oxysporum*

The PDA medium was supplemented with the required concentration of all parts of *C. lancifolius* and Fungicide Evaluation (25 mL, 50, and 100 mL each) before being solidified in a 250 mL Erlenmeyer flask, stirred, and poured into Petri dishes Sterilized. Three panels were assigned to each concentration and allowed to solidify. The other three plates of PDA medium served as controls. Petri dishes of each concentration were incubated at 25°C for 5 days. The growth diameter of the fungus was measured and calculated in centimeters 3, 4, and 5 days after inoculation.

Experimental design

The treatments were arranged in a Complete Randomized Block Design.

Statistical analyses

The obtained data were statistically analyzed according to the analysis of variance (ANOVA); Duncan's Multiple Range Test was used for mean separation.

RESULTS AND DISCUSSION

This study was conducted under laboratory conditions by Jica, College of Agricultural Studies, Sudan University of Science and Technology, Sudan, between November 2015 and February 2016 to investigate the inhibitory effect of all parts of aqueous extracts of *C. lancifolius* (leaves, bark, fruits, roots) and fungicide, achieving an efficacy of 250 EC against the growth of the fungus *F. oxysporum.*

Isolation and Identification from the infected sample

The isolation and identification of the fungus were based on the method of (Booth 1977) and on the colony's characteristics and microscopic examination. In addition, standard books and research papers were consulted in the search for these mushrooms (Aneja 2004).

Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after three days from inoculation

The results (Table 1, Table 2, and Figure 1) showed that all aqueous extracts of *C. lancifolius* (leaves, fruits, barks, roots) were screened, and the fungicidal effects on fungal growth had three days after inoculation. In addition, fungal growth inhibition was significantly elevated compared to the control.

Additionally, the highest concentration of plant extracts (100%) gave significantly higher inhibition than the untreated control, which gave (75.5%, 68%, 66%, and 50%). Of the tested parts of *C. lancifolius* extracts, the fruit was more effective in suppressing fungal growth than other parts of *C. lancifolius*, each producing (75.5) in (Table 1, Table 2), the results showed that the concentration of antifungal activity increases with increasing extract.

Table 1. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after three days from inoculation

Treat.	Conc.	Growth			
		R1	R2	R3	Mean
Leaves	25	4(2.1)	4.1(2.1)	4(2.1)	4.033(2.1)ab
	50	3.9(2.1)	4(2.1)	3.9(2.1)	3.93(2.1)ab
	100	2.5(1.7)	2.7(1.8)	3.1(1.9)	2.76(1.8)bc
Fruit	25	4(2.1)	4.1(2.1)	3.9(2.09)	4(2.09)ab
	50	2.05(1.6)	1.9(1.5)	2.2(1.6)	2.05(1.6(cd
	100	1.4(1.4)	1.2(1.3)	1.6(1.4)	1.4(1.36)d
Bark	25	3.5(2)	3.7(2)	3.3(1.7)	3.5(1.9)bc
	50	3(1.9)	2.5(1.7)	2.8(2)	2.76(1.8)bc
	100	1.6(1.4)	2(1.6)	1.7(1.4)	1.76(1.46)d
Root	25	3(1.9)	3.5(2)	3.7(2)	3.4(1.96)abc
	50	2.2(1.6)	2.6(1.8)	2.1(1.6)	2.3(1.66)cd
	100	1.7(1.4)	2.3(1.8)	2(1.6)	2(1.6)cd
Fungicide		0.3(0.9)	0(0.7)	0.4(0.7)	0.233(0.76)e
Control		5(2.3)	4.9(2.3)	5(2.3)	4.96(2.3)a
C.V				11.74	
SE				0.06	

Note: Means in the same column with the same letter (s) are not significant at P=0.05, according to DRMT. Values between brackets were transformed to √x+0.5

Table 2. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after five days from inoculation

Treatments	Conc.	Inhibition zone			
		R1	R2	R3	Mean
Leave	25	20.00	16.30	20.00	18.30
	50	22.00	18.30	22.00	20.70
	100	50.00	44.00	38.00	44.00
Fruit	25	20.00	16.30	22.00	19.40
	50	59.00	61.20	56.00	58.70
	100	72.00	75.50	68.00	71.80
Bark	25	30.00	24.40	34.00	29.50
	50	40.00	48.90	44.00	44.30
	100	68.00	59.18	66.00	64.40
Root	25	40.00	28.50	26.00	31.50
	50	56.00	49.00	58.00	54.30
	100	60.00	59.18	66.00	61.10
Fungicide		94.00	100.0	92.00	95.30
Control		00.00	00.00	00.00	00.00

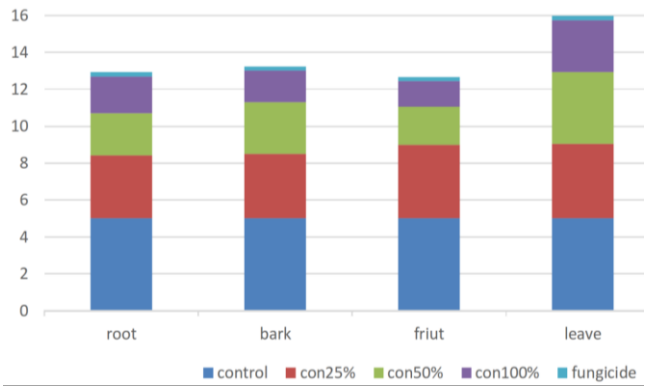

Figure 1. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after three days from inoculation

Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after four days from inoculation

Four days after inoculation, all parts of *C. lancifolius* plants tested concentrations, as well as that of the fungicide, invariably continued and showed inhibitory effects against fungal growth. However, the highest concentration of plant extracts (100%) gave the highest percentage of inhibition zones (68.57%, 42.85%, 57.14, and 55.71, respectively). This inhibitory effect of all concentrations tested significantly differed from the control (Table 3, Table 4, and Figure 2). Furthermore, the fruit part of the *C. lancifolius* plant extract remained the most suppressive at all tested concentrations, followed in descending order by the other parts.

Effect of different parts of *C. lancifolius* plant aqueous extracts and 4 on the linear growth of *F. oxysporum* after five days from inoculation

Within five days of inoculation, the results (Table 5, 6, and Figure 3) showed that extracts from all tested parts of *C. lancifolius* plants effectively suppress fungal growth. Indeed, all concentrations of all parts of *C. lancifolius* tested (100, 50, and 25%) induced significantly greater inhibition against the test fungus than the control, which gave (63.75, 43.75.59, 37, and 58.12%). Meanwhile, the aqueous fruit extract in high concentrations tested consistently showed a greater inhibitory effect than the other parts of the *C. lancifolius* aqueous plant extracts that give (63.75). It is clear that the organism under examination differs in its response to different concentrations of plant extracts, but in general, growth inhibition increases with concentration. This inhibitory effect of all concentrations was significantly different from the control.

Table 3. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after four days from inoculation

Treat.	Conc.	Growth			
		R1	R2	R3	Mean
Leave	25	5.75(2.5)	5.9(2.6)	5.75(2.5)	5.8(2.5)ab
	50	5.25(2.4)	5.7(2.5)	5.25(2.4)	5.4(2.4)bc
	100	3.75(2.1)	4(2.1)	4.1(2.1)	3.95(2.1)of
Fruit	25	4.5(2.2)	4.35(2.2)	4.5(2.2)	4.45(2.2)de
	50	2.9(1.8)	2.5(1.7)	2.9(1.8)	2.8(1.8)gh
	100	2.4(1.7)	2.2(1.6)	2.3(1.6)	2.3(1.63)h
Bark	25	5.8(2.5)	5.6(2.4)	5(2.3)	5.46(2.4)bc
	50	4.6(2.3)	4(2.1)	4.7(2.5)	4.43(2.3)cd
	100	3.0(1.9)	3.5(2)	3.3(1.9)	3.26(1.9)fg
Root	25	5.5(2.4)	5.7(2.5)	5.45(2.3)	5.6(2.4)bc
	50	3.5(2)	3.8(2)	3.9(2.1)	3.7(2.0)of
	100	3.1(1.9)	3.5(2)	3.5(2)	3.4(1.9)f
Fungicide		0.9(1.1)	0(0.7)	0.8(1.1)	0.56(0.96)i
Control		7.0(2.7)	7.0(2.7)	7.0(2.7)	7.0(2.7)a
C.V				4.71	
SE				0.07	

Note: Means in the same column with the same letter (s) are not significant at P=0.05, according to DRMT. Values between brackets were transformed to √x+0.5

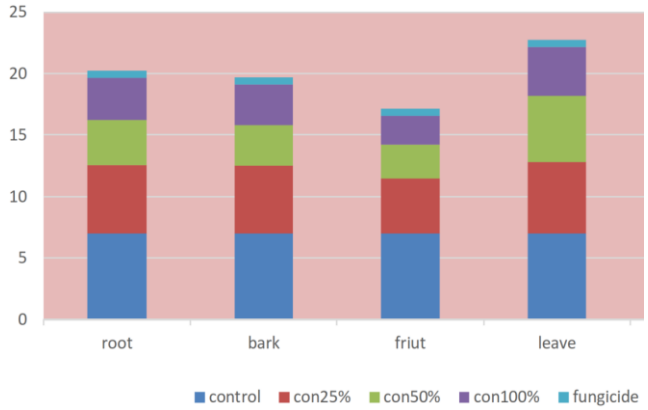

Figure 2. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after four days from inoculation

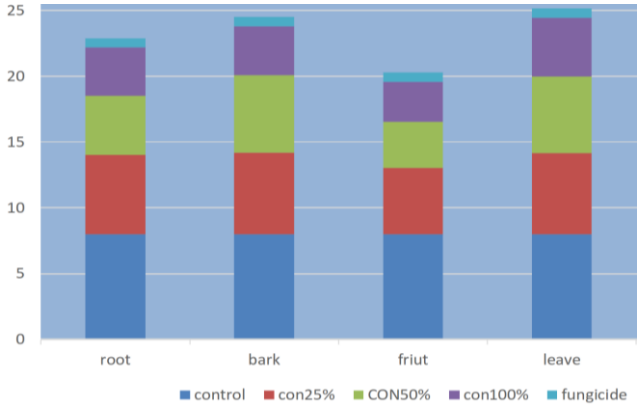

Figure 3. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after five days from inoculation

Table 4. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after four days from inoculation

Treatments	Cons.	Inhibition zone			
		R1	R2	R3	Mean
Leave	25	17.85	15.71	17.85	17.14
	50	25.00	18.5	25.00	22.83
	100	46.42	42.85	41.42	43.56
Fruit	25	35.7	37.8	35.7	36.40
	50	58.5	64.28	58.57	60.45
	100	65.71	68.57	68.24	67.50
Bark	25	17.14	20.00	28.57	21.90
	50	34.28	42.85	32.85	36.66
	100	57.14	50.00	52.28	53.14
Root	25	21.42	18.57	22.41	20.80
	50	50.00	45.71	44.28	46.66
	100	55.71	50.00	50.00	51.90
Fungicide		87.14	100.0	88.57	91.90
Control		00.00	00.00	00.00	00.00

Table 5. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after five days from inoculation

Treat.	Cons.	Growth			
		R1	R2	R3	R4
Leaves	25	6.15(2.6)	6.1(2.6)	6.2(2.6)	6.15(2.6)b
	50	5.56(2.5)	5.9(2.5)	5.8(2.5)	5.8(2.5)bc
	100	4.35(2.2)	4.5(2.2)	4.7(2.3)	4.51(2.2)d
Fruit	25	4.9(2.3)	4.9(2.3)	5.3(2.4)	5.0(2.3)cd
	50	3.7(2.05)	3.5(2)	3.35(2)	3.5(2.02)cd
	100	3(1.87)	2.9(1.8)	3.3(1.9)	3.06(1.85)f
Bark	25	6.65(2.6)	6.1(2.6)	6.4(2.6)	6.21(2.6)b
	50	6(2.5)	6(2.5)	5.7(2.5)	5.9(2.5)bc
	100	3.35(1.9)	4(2.1)	3.9(2.09)	3.71(2.03)of
Root	25	5.7(2.5)	6.25(2.6)	6.1(2.6)	6.016(2.56)b
	50	3.9(2.1)	4.5(2.2)	5(2.3)	4.46(2.2)de
	100	3.35(2)	4(2.1)	3.7(2)	3.68(2.03)ef
Fungicide		1.4(1.4)	0(0.7	0.8(1.1)	0.73(1.06)g
Control		8(2.9)	8(2.9)	8(2.9)	8(2.9)a

Note: Means in the same column with the same letter (s) are not significant at P=0.05, according to DRMT. Values between brackets were transformed to √x+0.5

Table 6. Effect of different parts of *C. lancifolius* plant aqueous extracts and fungicide on the linear growth of *F. oxysporum* after four days from inoculation

Treatments	Cons.	Inhibition zone			
		R1	R2	R3	Mean
Leave	25	23.12	23.75	22.50	23.12
	50	29.37	26.25	27.50	27.70
	100	45.62	43.75	41.25	43.54
Fruit	25	38.75	38.75	33.75	37.08
	50	53.75	56.25	58.12	56.04
	100	62.50	63.75	58.12	61.45
Bark	25	33.25	23.75	20.00	25.66
	50	25.00	25.00	28.75	26.25
	100	59.37	50.00	51.25	53.54
Root	25	28.75	21.87	23.75	24.79
	50	51.25	43.75	37.50	44.16
	100	58.12	50.00	53.75	53.95
Fungicide		82.50	100.0	90.00	90.83
Control		0.000	0.000	0.000	00.00

Discussion

The major pathogens of *Fusarium* wilt have a wide range of host plants and include numerous special forms, some contain two or more pathogenic breeds, causing devastating wilt diseases, and many are carried by seeds as listed by (Andersen 1974) for the following host's Alliums cannabis, Beta vulgaris, Cucumis sativa, Phaseolus vulgaris, and Psumist sativum.

The *F. oxysporum* is a major cause of wilting (Nene et al. 1991). The disease is common in most tomato-producing countries and is a major disease. It is a disease of seeds and soil. The fungal pathogen *F. oxysporum* affects a variety of hosts of different ages, such as tomatoes, tobacco, legumes, and cucurbits. Sweet potatoes, chickpeas, and bananas are among the most susceptible crops, but other herbaceous plants are also affected (Pan Germany 2010).

Several diseases are known to limit crop yields in Sudan. One of them is *Fusarium* wilt caused by *F. oxysporum*, which is one of the most important diseases causing economic losses (Bhatia et al. 2004). The disease is

considered especially severe in traditional production areas where crops are grown on stored soil moisture after the Nile floodwaters have subsided. As a result, farmers do not adhere to crop rotation in these areas, and the crop in the post-flowering phase is often subject to water stress during low tide years (Ali 1996).

A large body of research has identified various strategies to combat this fungal pathogen (Haware and Nene 1982). However, treating seed- and soil-borne diseases such as wilt caused by *F. oxysporum* has always been problematic (Haware and Kannaiyan 1992; Rao and Balachadran 2002). In general, synthetic fungicides greatly reduce the incidence of wilt, but their use is expensive and harmful to the environment (Song and Goodman 2001). Moreover, the use of resistant cultivars is fraught with resistance breakdown due to high pathogen variability in the pathogen population (Kutama et al. 2011). In this context, searching for an ecological way to control *Fusarium* wilt in crops that offer an alternative to fungicides is very challenging.

Historically, numerous phytochemicals isolated from various plants are now prescribed by physicians worldwide (Newman et al. 2000). Many plant extracts or products are as potent as many conventional synthetic pesticides and are effective at very low concentrations. On the other hand, botanical insecticides have great advantages over synthetic pesticides because they are more environmentally friendly and are accepted by most farmers, government organizations, and decision-makers.

In this study, the different parts of the aqueous extracts of the *C. lancifolius* plant were examined for their biological activity against *Fusarium* wilt. The data (Tables 1-3 and Figures 1-3) revealed that all screened aqueous extracts of *C. lancifolius* plants (leaves, fruits, bark, and root) consistently showed an inhibitory effect on fungal growth with a significantly high percentage of zones of inhibition. This result is in agreement with Satish et al. (1999), Ergene et al. (2006), Kiran and Raveesha (2006), Mohana and Raveesha (2006), Okigbo and Ogbonnaya (2006), and Sharif et al. (2006); who studied the effects of extracts from many higher plants and reported that they had antibacterial, antifungal and insecticidal properties in laboratory tests. More recent results have also been published by Saad et al. (2014), where they demonstrated the antibacterial and antifungal activities of the methanol extract of *C. lancifolius* air pieces using the disk diffusion method. Similar results were also obtained by Ahmed (2014), who used the alkaloid extract of *C. lancifolius* against certain clinical pathogens. From this study, it can be concluded that: (i) the extracts of leaves, fruits, bark, and roots of the *C. lancifolius* plant tested showed an inhibitory effect on the growth of fungi. *F. oxysporum* This more fungicidal component (score) could be applied as part of an integrated approach to control *Fusarium* wilt. (ii) Of the plant parts of *C. lancifolius*, the aqueous extract in high concentration showed an inhibitory effect compared to the others. (iii) The screened concentrations of all parts of *C. lancifolius* aqueous extracts differ in their responses to fungal assays. Likewise, the test organism reacted differently to different concentrations of extracts. This variability in the response expressed by the test organism can be used to adjust an optimal dose to control *Fusarium* wilt.

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